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A METHOD FOR INHIBITING IMMUNOGLOBULIN-INDUCED TOXICITY RESULTING FROM THE USE OF IMMUNOGLOBULINS IN THERAPY AND IN VIVO DIAGNOSIS

Abstract:

Abstract of WO9805787

The present invention provides a method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy in a subject comprising administering an immunoglobulin or Ig fusion protein molecule to the subject, the immunoglobulin molecule having a variable region and a constant region, the immunoglobulin molecule being modified prior to administration by inactivation of at least a portion of the constant region.

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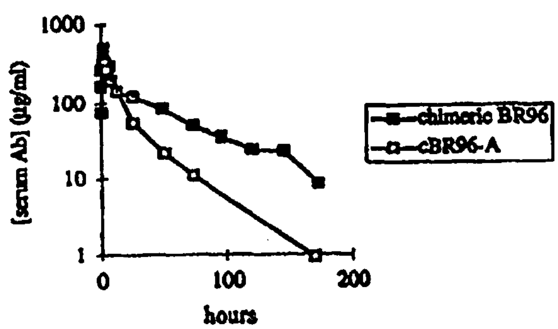

Plasma clearance in high LeY expressing dogs chimeric versus constant region mutant of cBR96-2.

(57) Abstract

The present invention provides a method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy in a subject comprising administering an immunoglobulin or Ig fusion protein molecule to the subject, the immunoglobulin molecule having a variable region and a constant region, the immunoglobulin molecule being modified prior to administration by inactivation of at least a portion of the constant region.

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A METHOD FOR INHIBITING IMMUNOGLOBULIN-INDUCED TOXICITY RESULTING FROM THE USE OF IMMUNOGLOBULINS IN THERAPY AND IN VIVO DIAGNOSIS

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to methods for inhibiting or reducing immunoglobulin-induced toxicity resulting from therapy or in vivo diagnosis. Specifically, in lieu of using unmodified antibodies or recombinant binding proteins for in vivo use, the invention provides the use of modified antibodies or recombinant binding proteins which have been structurally altered in the constant domain so that upon administration immunoglobulin-induced toxicity is reduced or inhibited.

BACKGROUND OF THE INVENTION

Over the years investigators have attempted to harness the immune system for therapeutic use. Immunoglobulin (Ig) molecules which constitute an important part of the immune system are of great interest because they (1) react with a diverse family of ligands, (2) possess different effector functions and (3) are of great biological importance. Despite its potential, a persistent problem with

immunoglobulin immunotherapy has been, among other problems, the toxic effect to normal cells of using antibodies which recognize both normal and diseased cells. This problem is far-reaching because the majority of antibodies presently available recognize a target located on both normal and diseased cells (Slavin-Chiorini, et al., Int. J. Cancer 53: 97-103 (1993)).

The constant region can promote cell death through antibody dependent cell mediated cytotoxicity (ADCC) or by complement dependent cytotoxicity (CDC). Despite the deletion of portions of the constant region, particularly the CH_2 domain, the antigen binding function can be retained (D. Yelton, M. Scharf, Mutant monoclonal antibody with alterations in biological functions, J. Exp. Methods 156:1131-1148 (1982)).

Others have generated a CH_2-deleted antibody (Mueller et al., Proc. Natl. Acad. Sci. USA 87: 5702-5705 (1990)). Their findings provide that the CH_2-deleted antibody was cleared from the blood of tumor-bearing mice much faster than the corresponding intact antibody. Other in vivo findings also confirmed that a CH_2-deleted antibody, designated ch14.18DCH2, is a potentially useful reagent for radioimmunodetection of human tumors because of its reduced immunogenicity, increased target specificity, and rapid clearance from circulation (Mueller et al., Proc. Natl. Acad. Sci. USA 87: 5702-5705 (1990).

Generally, whole antibody molecules are composed of two heavy (H) and two light (L) chains which are held together by covalent bonds (disulfide) and non-covalent interactions. Each chain contains a variable region (V) and a constant region (C). The variable regions at the amino termini of the two chains form the antigen binding region. The constant region of the H chain has three components or domains. Occasionally, the first constant region domain (CH_1) interacts with the C region of the L chain through hydrophobic interactions and generally a disulfide bond,

depending on isotype. The next C region stretch is the hinge-acting disulfide bond stably introduced between two H chains. The second constant region domain (CH_2) is adjacent to the hinge region. CH_2 contains sequences important for effector functions of the antibody, such as the sequences responsible for complement fixation, and Fc receptor binding The third constant region domain (CH_3) is located at the carboxyl terminus of the H chain, and is considered to play an important role in H chain assembly as well as some C region functions.

Today many antibodies in clinical trials are directed against tumor associated antigens. Most tumor associated antigens are not tumor specific but are also generally found on the cell surface of some normal, non-tumorigenic cells. The clinical use of some antibodies directed against tumor associated antigens are limited because of the toxicity associated with their use. Therefore, there is a need for methods for inhibiting toxicity associated with immunoglobulin use in the field of disease therapy (e.g., therapy for tumors, kidney disease, and the like) and in vivo diagnosis.

We addressed this need by discovering methods for inhibiting or reducing toxicity to normal cells generally associated with immunoglobulin immunotherapy or in vivo diagnosis, wherein the immunoglobulin recognizes both diseased and normal cells. Our discovery involves generating immunoglobulin molecules or Ig fusion proteins having structurally altered constant regions which inhibit or reduce immunoglobulin-induced toxicity.

SUMMARY OF THE INVENTION

The present invention provides methods for inhibiting immunoglobulin-induced toxicity by using known immunoglobulin or Ig fusion protein molecules which are structurally altered in their constant regions so that the resulting structurally altered

immunoglobulin or Ig fusion protein molecules exhibit reduced or inhibited toxicity in vivo compared to their original unmodified counterparts.

Structural alteration of the constant region may be effected in a number of ways as long as it results in reducing or inhibiting immunoglobulin-induced toxicity.

In accordance with the practice of the invention, structural alteration of the constant region is effected by deletion of the entire constant region. In another embodiment, only the CH_2 domain is deleted. In another embodiment, only that portion of the CH_2 domain that binds the Fc receptor is deleted. In yet another embodiment, only that portion of the CH_2 domain that binds the complement component C1q is deleted. Alternatively, in another embodiment, multiple deletions in discrete Fc receptor and complement component binding domains are effected.

Alternatively, structural alteration is effected by single or multiple mutations in the CH_2 domain such as amino acid insertions and substitutions. The mutation or mutations must result in inhibiting immunoglobulin-induced toxicity. By way of example, the amino acids in multiple toxicity associated domains in the constant region can be altered so as to render the constant region unable to mediate a ADCC response or activate complement thereby inhibiting immunoglobulin induced toxicity resulting from immunotherapy. Alternatively, multiple amino acids in a single toxicity associated domain in the constant region can be altered.

Further alternatively, structural alteration can be effected by isotype switching resulting in an altered immunoglobulin molecule that either does not induce toxicity or induces some limited toxicity but does not cause a harmful effect. For example, isotype switching can result in the constant region being unable to mediate a CDC or ADCC response or some other activity which mediates toxicity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a line graph showing plasma clearance in high Le^Y expressing dogs using chimeric BR96 versus constant region mutant of cBR96-2.

Figure 2 is a schematic diagram of a plasmid designated pTWD-cJVK.L1 including the chimeric (c)BR96-light chain (SEQ ID NO. 11).

Figure 3 is a schematic diagram of a plasmid designated pD16hJ1.L1 including the human (h)BR96-light chain (SEQ ID NO. 13).

Figure 4 is a schematic diagram of a plasmid, designated pD17-hJm14-dCH2.H1, of hBR96-2A (i.e., human mutant BR96 having the H1, H2, and H3 mutations and the CH_2 deletion (PCT Application No. 95/305444, published March 6, 1996)).

Figure 5 is a schematic diagram of a plasmid, designated pD17-cJ-dCH2.H1, of cBR96-A (SEQ ID NO. 10) (i.e., chimeric BR96 having the CH_2 deletion (PCT Application No. 95/305444, published March 6, 1996)).

Figure 6 is a schematic diagram of a plasmid, designated pD17-cJ.H1, of cBR96.

Figure 7 is a line graph showing the results of an ELISA assay of (1) hBR96-2A-Dox to Le^y (closed diamond), (2) hBR96-2A to Le^y (96:0006A2 R/A)(closed square), (3) hBR96-2A to Le^y (96:0006B R/A)(closed triangle), and BR96-Dox to Le^y (X).

Figure 8 is a line graph showing the results of an ELISA assay of (1) BR96-A-Dox to Le^y (closed diamond), (2) chiBR96 to Le^y (closed square), (3) cBR96-A to Le^y (96:0003 R/A)(closed triangle), and cBR96-Dox to Le^y (X).

Figures 9a-c are schematic diagrams showing the steps for deleting a CH_2 domain.

Figures 10a-c are schematic diagrams showing the construction of BR96 IgG1 CH_2 domain point mutations.

Figure 11 is a schematic diagram showing the construction of the pNg1/14 vector.

Figure 12 is a schematic diagram showing the construction of pD17-hBR96-2.

Figure 13 is a schematic diagram showing the construction of pD17-hJm14-dCH2.H1.

Figure 14 is the nucleic acid sequence of pD17-cJ-dCH2.H1, the plasmid shown in Figure 5, chimeric BR96 having the CH_2 deletion.

Figure 15 is a line graph showing the results of an ELISA assay comparing whole chiBR96 and deleted CH_2 chiBR96 on Le^y.

Figure 16 is a description of the seven structural alterations.

Figure 17 is a schematic diagram of a plasmid designated pD17-hG1b.

Figure 18 is the nucleic acid sequence of pD17-hJm14.H1.

Figure 19 is the nucleic acid sequence of pD17-hG1b.

Figure 20 is a line graph showing complement dependent cytotoxicity. In the legend, the closed square is hBR96-1; closed diamond is hBR96-2B; closed circle is

hBR96-2C; closed triangle is hBR96-2D; open square is hBR96-2H; open circle is hBR96-2A and open triangle is 2B8, anti-*Pseudonomas aeruginosa* flagella type b mAb, negative control.

Figure 21 is a line graph showing antibody dependent cell-mediated cytotoxity. In the legend, the closed square is hBR96-1; closed diamond is hBR96-2B; closed circle is hBR96-2C; closed triangle is hBR96-2D; open square is hBR96-2H; open circle is hBR96-2A and open triangle is 2B8, anti-*Pseudonomas aeruginosa* flagella type b monoclonal antibody (mAb), negative control.

Figure 22 is a line graph showing binding activity of hBR96-2 constant region mutants on LeY-HSA. In the legend, the solid diamond is hBR96-1; solid square is hBR96-2A (CH2 deletion); solid triangle is hBR96-2B (235, 237 mutations); open square is hBR96-2C (318, 320, 322 mutations); open circle is hBR96-2D (331 mutation); and open triangle is hBR96-2H (235, 237, 318, 320, 322, 331 mutations).

Figure 23 is a line graph showing binding activity of hBR96-2 constant region mutants on LNFPIII-BSA. LNFPIII is a lacto-N-fucopentasose, a Lewis X trisaccharide with an additional lactose spacer (V Labs, Covington, LA). In the legend, the solid diamond is hBR96-1; solid square is hBR96-2A (CH2 deletion); solid triangle is hBR96-2B (235, 237 mutations); open square is hBR96-2C (318, 320, 322 mutations); open circle is hBR96-2D (331 mutation); and open triangle is hBR96-2H (235, 237, 318, 320, 322, 331 mutations).

Figures 24A and 24B provide a strategy for introducing multiple mutations by RPCR. (A) Diagram of he 1.4 kpb IgG heavy chain region showing the hinge CH_2 and CH_3 domains as boxed regions. Site-specific mutations to be introduced into CH_2 positions L1, L2, and L3 are encoded by complementary sets of mutant PCR

primers (A1 and A2; B1 and B2; and C1 and C2). The asterisks (*) indicate the number of amino acid changes introduced at each L position. The two PCR primers, Rs (Recombination -sense) and Ra (Recombination-antisense), flank the Eco-47-III restriction sites and mediate homologous recombination with vector ends. The 3' ends of the oligonucleotides are represented by arrowheads. (B) A three-way homologous recombination event between fragments RsA2, A1Ra and the linearized vector produces the L1 mutant IgG. Two distally located sets of mutations (L1 and L2) are simultaneously introduced by increasing the number of recombining PCR produces as is shown in the four-way recombination of RsA2, A1B1, B1Ra with vector.

Figure 25 is a gel showing Eco-47-III restriction endonuclease analysis of DNAs prepared from colonies generated by multiple PCR fragment RPCR. Lane M: 1kb ladder DNA marker (GIBCO/BRL Life Science Technology). Lanes 1-12: Twelve randomly selected colonies resulting from quadruple homologous recombination events were used to prepare plasmid and digested with Eco47-III. Clones 1, 2, 6 and 9 contain the fully assembled 1.4 kpb insert.

Figure 26 provides the amino acid sequence for hBR96-2 heavy-chain variable region and the human IgG1 constant region.

Figure 27 provides the amino acid sequence for hBR96-2A heavy-chain variable region and the human IgG1 constant region.

Figure 28 provides the amino acid sequence for chi BR96 heavy-chain variable region and the human IgG1 constant region without the CH_2 domain.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

As used herein the term "inhibiting immunoglobulin-induced toxicity" means to reduce or alleviate symptoms generally associated with toxicity caused by immunoglobulin or Ig fusion protein therapy, e.g., toxicity mediated by effector functions of the Fc receptor. For example, BR96 antibody recognizes and binds BR96 antigen which is found at some levels in the gastrointestinal tract and at elevated levels in tumors (as compared to the gastrointestinal tract of normal tissues). The binding of BR96 antibody to BR96 antigen in vivo causes symptoms associated with gastrointestinal toxicity. These symptoms include rapid onset of vomiting, often with blood, and nausea. In humans the bleeding is limited to the fundus of the stomach, causing erosion of the superficial mucosa of the stomach.

The pathology of the wound is limited and resolves. However, the extreme nature of the nausea and vomiting, unrelieved by anti-emetics, defines it as the dose-limiting toxicity. For highly elevated levels of other antigens found in the central nervous system (CNS), liver, and other locations, the toxicity will be characterized by symptoms other than those described above.

As used herein the term "immunoglobulin molecule" can be produced by B cells or be generated through recombinant engineering or chemical synthetic means. Examples of immunoglobulin molecules include (1) antibodies, e.g., polyclonal and monoclonal antibodies, chimeric or humanized, and (2) recombinant Ig containing binding proteins, e.g., Ig fusion proteins. Recombinant Ig containing binding proteins include cell surface proteins, e.g., CD antigens (in one embodiment, CTLA4), to which an Ig tail is joined.

As used herein the terms "structurally altered" or "structural alteration" means manipulating the constant region so that the resulting molecule or protein exhibits a diminished ability to induce toxicity. Structural alteration can be by chemical modification, proteolytic alteration, or by recombinant genetic means. Recombinant genetic means may include, but is not limited to, the deletion, insertion and substitution of amino acid moieties.

As used herein the terms "multiple toxicity associated domains" means more than one discrete toxicity associated domain. As there appear to be at least two toxicity associated domains in the immunoglobulin molecule, one roughly localized to amino acids 231-238 and another roughly localized to amino acids 310-331, an example of the structural alteration of multiple toxicity associated domains comprises the insertion, substitution or deletion of amino acid residues in both of these domains. This definition excludes structural alterations targeting a single toxicity associated domain.

Merely by way of example, the constant region of the immunoglobulin molecule can be structurally altered so that the molecule no longer mediates a CDC or ADCC response. However, the methods of the invention encompasses the use of structurally altered immunoglobulin molecules regardless of whether it mediates a CDC or ADCC response. The underlying requirement is that the altered molecule must inhibit immunoglobulin-induced toxicity.

Structural alteration can be effected in a number of ways. For example, structural alteration can be effected by deletion of the entire constant region.

Alternatively, structural alteration can be effected by deletion of the entire CH_2 domain of the constant region. In this instance, deletion of the entire CH_2 domain may render the molecule unable to (1) bind an Fc receptor thereby eliminating the

molecule's possibility of mediating antibody-dependent cellular cytotoxicity (ADCC), (2) bind C1q, or (3) activate complement.

Alternatively, structural alteration can be effected by deletion of only that portion of the CH_2 domain that binds the Fc receptor or complement.

Further alternatively, a single mutation or multiple mutations such as substitutions and insertions in the CH_2 domain can be made. The underlying requirement of any mutation is that it must inhibit, diminish, or block immunoglobulin-induced toxicity. For example, this can be achieved by mutating the constant region such that the altered molecule is rendered unable to mediate a CDC response or an ADCC response, or to activate complement.

Alternatively, structural alteration can be effected by isotype switching (also known as class switching) so that the altered molecule does not induce toxicity in the subject. In one embodiment, the constant region of the immunoglobulin is structurally altered so that it no longer binds the Fc receptor or a complement component, e.g., switching a molecule's original IgG isotype from IgG1 to IgG4. Isotype switching can be effected regardless of species, i.e., an isotype from a non-human being can be switched with an isotype from a human being (E.D. Finkelman et al. (1990) Annu. Rev. Immunol. 8:303-333; T. Honjo et al. (1979) Cell 18: 559-568; T. Honjo et al. In "Immunoglobulin Genes" pp. 124-149 Academic Press, London)).

As used herein the term "Ig fusion protein" means any recombinantly produced antigen or ligand binding domain having a constant region which can be structurally altered.

As used herein "cytotoxic agent" includes antimetabolites, alkylating agents, anthracyclines, antibiotics, anti-mitotic agents, and chemotherapeutic agents. Specific examples within these groups include but are not limited to ricin, doxorubicin, daunorubicin, taxol, ethidium bromide, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, supporin, gelonin, PE40, bryodin, dihydroxy anthracin dione, actinomycin D, and 1-dehydrotestosterone.

As used herein the term "BR96" refers to (1) the whole BR96 monoclonal antibody disclosed in PCT No. 95/305444, published March 6, 1996, (2) chimeric BR96 monoclonal antibody disclosed in PCT No. 95/305444, published March 6, 1996, or (3) BR96 mutant molecules disclosed in PCT No. 95/305444, published March 6, 1996.

As used herein, "treating" means to (1) provide tumor regression so that the tumor is not palpable for a period of time (standard tumor measurement procedures may be followed (A.B. Miller et al. "Reporting results of cancer treatment" Cancer 47:207-214 (1981)); (2) stabilize the disease; or (3) provide any clinically beneficial effects.

As used herein, an "effective amount" is an amount of the antibody, immunoconjugate, or recombinant molecule which kills cells or inhibits the proliferation thereof.

As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular or subcutaneous administration, or the implantation of a slow-release device such as a miniosmotic pump, to the subject.

As used herein, "pharmaceutically acceptable carrier" includes any material which when combined with the antibody retains the antibody's specificity or efficacy and is

non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

As used herein, "mutation" means a single amino acid or nucleic acid mutation or multiple mutations by whatever means, e.g., homologous recombination, error prone PCR, or site directed mutagenesis.

In order that the invention herein described may be more fully understood, the following description is set forth.

METHODS OF THE PRESENT INVENTION

The present invention provides a method for inhibiting immunoglobulin-induced toxicity resulting from the use of immunoglobulin during therapy or in vivo diagnosis. For example, the methods of the invention would be useful to minimize the toxicity associated with prolonged clinical exposure to immunoglobulin use during or after tumor imaging with radiolabeled antibodies.

In accordance with the practice of this invention, the subject includes, but is not limited to, human, equine, porcine, bovine, murine, canine, feline, and avian

subjects. Other warm blooded animals are also included in this invention.

This method comprises administering an immunoglobulin molecule to the subject. The immunoglobulin can be IgG, IgM, or IgA. IgG is preferred.

In one embodiment of the invention, the immunoglobulin molecule recognizes and binds Le^y. In another embodiment, the immunoglobulin recognizes and binds Le^x. In a further embodiment, the immunoglobulin is a monoclonal antibody BR96 produced by the hybridoma deposited on February 22, 1989 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 and accorded ATCC Accession No.: HB 10036. In yet another embodiment, the immunoglobulin is a chimeric antibody ChiBR96 produced by the hybridoma deposited on May 23, 1990, with the ATCC, 12301 Parklawn Drive, Rockville, MD 20852 and accorded ATCC Accession No.: HB 10460.

In accordance with the practice of the invention, the immunoglobulin can be a bispecific antibody with a binding specificity for two different antigens, one of the antigens being that with which the monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC binds. Also, in accordance with the practice of the invention, the immunoglobulin can be an anti-idiotypic antibody.

As required by the invention, at least a portion of the constant region of the immunoglobulin molecule is structurally altered. Structural alteration can be effected by a number of means. In one embodiment, the entire constant region, i.e., CH_1, CH_2, and CH_3 domains, can be deleted.

In another embodiment, only the CH_2 domain is deleted from the immunoglobulin molecule (e.g., cBR96-A (Figure 5), hBR96-2A (Figure 4). In this embodiment, the

CH_2 deletion may result in a molecule unable to bind the Fc receptor or a complement component.

In another embodiment, only that portion of the CH_2 domain which binds the complement component C1q is deleted. In yet another embodiment, mutations in specific portions of the CH_2 domain are made. For example, the immunoglobulin molecule may be modified by structurally altering multiple toxicity associated domains in the constant region so that immunoglobulin-induced toxicity is inhibited. A discussion of such mutations are further found hereinafter.

Regardless of the means, the underlying requirement for any structural alteration of the constant region is that immunoglobulin-induced toxicity is substantially reduced or inhibited. In one embodiment, immunoglobulin-induced toxicity is inhibited by structurally altering the constant region such that the molecule's ability to mediate a CDC response or ADCC response and/or activate the complement cascade is prevented or inhibited. Methods for determining whether the molecule is able to inhibit a CDC response are well known, e.g., one method involves a ^{51}Cr-release test (H. Garrigues et al. Int. J. Cancer 29:511 (1982); I. Hellström et al. PNAS 82:1499 (1985)). Methods for determining whether the molecule is able to inhibit an ADCC response are well known (I. Hellström et al. PNAS 82:1499 (1985)). Methods for determining whether the molecule is able to activate a complement cascade are well known.

In another embodiment of the invention, the method comprises administering to the subject an Ig fusion protein having a structurally altered constant region. Structural alteration of the constant region may include deletion of the entire C region or portions thereof, e.g., alteration of the CH_2 domain so that the altered molecule no longer binds the Fc receptor or a complement component.

The invention further provides a method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy in a subject. The method comprises administering to the subject an antibody which has been modified so that at least a portion of the constant region has been structurally altered as discussed supra. In one embodiment, the antibody recognizes and binds Le^y. In another embodiment, the antibody recognizes and binds to Le^x.

In accordance with the practice of this invention, the antibody can be monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC. Alternatively, the antibody can be chimeric antibody ChiBR96 produced by the hybridoma having the identifying characteristics of HB 10460 as deposited with the ATCC. Further, the antibody can be a bispecific antibody with a binding specificity for two different antigens, one of the antigens being that with which the monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC binds.

Additionally, the present invention provides a method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy for a disease in a subject. The disease will vary with the antigen sought to be bound. Examples of diseases include but are not limited to immunological diseases, cancer, cardiovascular diseases, neurological diseases, dermatological diseases or kidney disease.

This method comprises the following steps. Step one provides selecting an antibody for a target. Generally, the target is associated with the disease and the antibody directed to the target is known. For example, the target can be the BR96 antigen and the antibody selected is BR96.

Step two of this method provides structurally altering the constant region of the antibody so selected so that immunoglobulin induced toxicity is inhibited. Inactivation can include any of the means discussed above. For example, inactivation can be effected by structurally altering multiple toxicity associated domains in the CH_2 domain of the constant region of the Ig protein so selected.

Step three of this method provides administering the structurally altered antibody of step two to the subject under conditions that the structurally altered antibody recognizes and binds the target and that such binding directly or indirectly alleviates symptoms associated with the disease.

In accordance with the invention, in one embodiment step one provides selecting an Ig fusion protein for a target. Further, the method provides mutating the Ig fusion protein so selected by structurally altering the CH_2 domain of the constant region of the Ig protein by the same means discussed above.

The invention further provides methods to treat human carcinoma. For example, the immunoglobulin, antibody, or Ig fusion protein discussed above can be used in combination with standard or conventional treatment methods such as chemotherapy, radiation therapy or can be conjugated or linked to a therapeutic drug, or toxin, as well as to a lymphokine or a tumor-inhibitory growth factor, for delivery of the therapeutic agent to the site of the carcinoma.

Techniques for conjugating therapeutic agents to immunoglobulins are well known (see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellström et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In

Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982)).

Alternatively, the structurally altered antibody or Ig fusion protein can be coupled to high-energy radiative agents, e.g., a radioisotope such as ^{131}I; which, when localized at the tumor site, results in a killing of several cell diameters (see, e.g., Order, "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985)). According to yet another embodiment, the structurally altered BR96 antibody can be conjugated to a second antibody to form an antibody heteroconjugate for the treatment of tumor cells as described by Segal in United States Patent 4,676,980.

Still other therapeutic applications for the structurally altered antibody or Ig fusion protein of the invention include conjugation or linkage, e.g., by recombinant DNA techniques or protein chemical techniques, to an enzyme capable of converting a prodrug into a cytotoxic drug and the use of that antibody-enzyme conjugate in combination with the prodrug to convert the prodrug to a cytotoxic agent at the tumor site (see, e.g., Senter et al., "Anti-Tumor Effects Of Antibody-alkaline Phosphatase", Proc. Natl. Acad. Sci. USA, 85:4842-46 (1988); "Enhancement of the in vitro and in vivo Antitumor Activities of Phosphorylated Mitomycin C and Etoposide Derivatives by Monoclonal Antibody-Alkaline Phosphatase Conjugates", Cancer Research 49:5789-5792 (1989); and Senter, "Activation of Prodrugs by Antibody-Enzyme Conjugates: A New Approach to Cancer Therapy," FASEB J. 4:188-193 (1990)).

It is apparent therefore that the present invention encompasses pharmaceutical compositions including immunoglobulin molecules, antibodies, and Ig fusion proteins all having structurally altered CH_2 domains, and their use in methods for treating human carcinomas. For example, the invention includes pharmaceutical compositions for use in the treatment of human carcinomas comprising a pharmaceutically effective amount of a structurally altered BR96 and a pharmaceutically acceptable carrier.

The compositions may contain the structurally altered antibody or Ig fusion protein or antibody fragments, either unmodified, conjugated to a therapeutic agent (e.g., drug, toxin, enzyme or second antibody). The compositions may additionally include other antibodies or conjugates for treating carcinomas (e.g., an antibody cocktail).

The compositions of the invention can be administered using conventional modes of administration including, but not limited to, intrathecal, intravenous, intraperitoneal, oral, intralymphatic or administration directly into the tumor. Intravenous administration is preferred.

The composition of the invention can be in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application.

The compositions of the invention also preferably include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate.

In accordance with the practice of the invention, the pharmaceutical carrier can be a lipid carrier. The lipid carrier can be a phospholipid. Further, the lipid carrier can be a fatty acid. Also, the lipid carrier can be a detergent. As used herein, a detergent is any substance that alters the surface tension of a liquid, generally lowering it.

In one example of the invention, the detergent can be a nonionic detergent. Examples of nonionic detergents include, but are not limited to, polysorbate 80 (also known as Tween 80 or (polyoxyethylenesorbitan monooleate), Brij, and Triton (for example Triton WR-1339 and Triton A-20).

Alternatively, the detergent can be an ionic detergent. An example of an ionic detergent includes, but is not limited to, alkyltrimethylammonium bromide.

Additionally, in accordance with the invention, the lipid carrier can be a liposome. As used in this application, a "liposome" is any membrane bound vesicle which contains any molecules of the invention or combinations thereof.

The most effective mode of administration and dosage regimen for the compositions of this invention depends upon the severity and course of the disease, the patient's health and response to treatment and the judgment of the treating physician.

The interrelationship of dosages for animals of various sizes and species and humans based on mg/m^2 of surface area is described by Freireich, E.J., et al. Cancer Chemother., Rep. 50 (4): 219-244 (1966). Adjustments in the dosage regimen can be made to optimize the tumor cell growth inhibiting and killing response, e.g., doses can be divided and administered on a daily basis or the dose reduced proportionally depending upon the situation (e.g., several divided doses can be

administered daily or proportionally reduced depending on the specific therapeutic situation).

THE MOLECULES OF THE INVENTION

The present invention provides structurally altered BR96 or BR96 Ig fusion proteins. Structurally altered BR96 antibodies or Ig fusion proteins have the variable region of BR96 and a modified constant region. This modification provides structurally altered BR96 antibodies or Ig fusion proteins with the ability to inhibit immunoglobulin-induced toxicity.

Various embodiments of structurally altered BR96 or BR96 Ig fusion proteins have been made.

In one embodiment, designated cBR96-A, the entire CH_2 domain of cBR96 was deleted. CBR96-A is expressed by the plasmid having the sequence shown in SEQ. ID. NO. 10. cBR96 is expressed by a plasmid having the sequence in SEQ ID NO. 9.

In another embodiment, designated hBR96-2A, the entire CH_2 domain of hBR96 was deleted. hBR96-A is expressed by the plasmid having the sequence shown in SEQ. ID. NO. 12. hBR96 is a mutant BR96 having the H1, H2, and H3 mutations described in PCT Application No. 95/305444, published March 6, 1996.

In yet another embodiment, designated hBR96-2B, the leucine residue located at amino acid position 235 is mutated to alanine. Additionally, the glycine residue located at amino acid position 237 is mutated to alanine. The amino acid position numbering used is described in Kabat et al. Sequences of Proteins of Immunological Interest 5th Edition (1991) United States Department of Health and Human Services.

In a further embodiment, designated hBR96-2C, the glutamic acid residue at position 318 is mutated to serine; the lysine residue located at position 320 is mutated to serine; and the lysine residue located at position 322 is mutated to serine using standard protocols (Alexander R. Duncan and Greg Winter "The binding site for C1q on IgG" Nature 332:738 (1988)).

In another embodiment, designated hBR96-2D, the proline residue at position 331 is mutated to alanine (M-H. Tao et al., "Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation" J. Exp. Med. 178:661-667 (1993); Y. Xu et al., "Residue at position 331 in the IgG1 and IgG4 domains contributes to their differential ability to bind and activate complement" J. Biol. Chem. 269:3469-3474 (1994)).

In an additional embodiment, designated hBR96-2E, the leucine residue at position 235 is mutated to alanine; the glycine residue located at position 237 is mutated to alanine; the glutamic acid residue located at position 318 is mutated to serine; the lysine residue located at position 320 is mutated to serine; and the lysine residue located at position 322 is mutated to serine (A. Morgan et al., "The N-terminal end of the CH_2 domain of chimeric human IgG1 anti-HLA-DR is necessary for C1q, Fc(gamma)RI and Fc(gamma)RIII binding" Immunol. 86:319-324 (1995)).

In yet a further embodiment, designated hBR96-2F, the leucine residue located at position 235 is mutated to alanine; the glycine residue located at position 237 is mutated to alanine; and the proline residue located at position 331 is mutated to alanine.

In yet another embodiment, designated hBR96-2G, the glutamic acid residue located at position 318 is mutated to serine; the lysine residue located at position 320 is

mutated to serine; the lysine residue located at position 322 is mutated to serine; and the proline residue located at position 331 is mutated to alanine.

In another embodiment, designated hBR96-2H, the leucine residue located at position 235 is mutated to alanine; the glycine residue located at position 237 is mutated to alanine; the glutamic acid residue at position 318 is mutated to serine; the lysine residue located at position 320 is mutated to serine; the lysine residue located at position 322 is mutated to serine; and the proline residue located at position 331 is mutated to alanine.

Depending on its form, a structurally altered BR96 antibody or fusion protein can be a monofunctional antibody, such as a monoclonal antibody, or bifunctional antibody, such as a bispecific antibody or a heteroantibody. The uses of structurally altered BR96, i.e., as a therapeutic or diagnostic agent, will determine the different forms of structurally altered BR96 which is made.

Several options exists for antibody expression. Immunoexpression libraries can be combined with transfectoma technology, i.e., the genes for the Fab molecules derived from the immunoglobulin gene expression library can be connected to the desired constant-domain exons. These recombinant genes can then be transfected and expressed in a transfectoma that would secrete an antibody molecule.

Once produced, the polypeptides of the invention can be modified, i.e., by amino acid modifications within the molecule, so as to produce derivative molecules. Such derivative molecules would retain the functional property of the polypeptide, namely, the molecule having such substitutions will still permit the binding of the polypeptide to the BR96 antigen or portions thereof.

It is a well-established principle of protein chemistry that certain amino acid

substitutions, entitled "conservative amino acid substitutions," can frequently be made in a protein without altering either the conformation or the function of the protein.

Amino acid substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative".

Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa.

Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine and valine (V).

Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

In one embodiment of the present invention, the polypeptide is substantially pure, i.e., free of other amino acid residues which would inhibit or diminish binding of the polypeptide to its target and would inhibit or reduce gastrointestinal toxicity which are normally exhibited during or after antibody therapy.

NUCLEIC ACID MOLECULES ENCODING THE PRESENT INVENTION

The nucleotide sequences and the amino acid sequences of the variable and constant regions of BR96 are known. The sequence for the immunoglobulin constant region is known and provided in Figure 18. Specific mutations in the constant region of the BR96 antibody were made. Nucleic acid molecules encoding the seven mutants described above (hBR96-2B through hBR96-2H) are as follows.

In hBR96-2B, alanine at amino acid positions 235 and 237 is encoded by codons GCU, GCC, GCA, or GCG.

In hBR96-2C, serine at positions 318, 320, and 322 is encoded by UCU, UCC, UCA, or UGG.

In hBR96-2D, alanine at position 331 is encoded by codons GCU, GCC, GCA, or GCG.

In hBR96-2E, alanine at positions 235 and 237 is encoded by codons GCU, GCC, GCA, or GCG. Serine at positions 318, 320, and 322 is encoded by UCU, UCC, UCA, or UGG.

In hBR96-2F, alanine at positions 235, 237, and 331 is encoded by codons GCU, GCC, GCA, or GCG.

In hBR96-2G, serine at positions 318, 320, 322 is encoded by UCU, UCC, UCA, or UGG. Further, the alanine at position 331 is encoded by codons GCU, GCC, GCA, or GCG.

In hBR96-2H, alanine at positions 235, 237, and 331 is encoded by codons GCU,

GCC, GCA, or GCG. Additionally, serine at positions 318, 320, 322 is encoded by UCU, UCC, UCA, or UGG.

Any of the above can be deoxyribonucleic acid (DNA), e.g., complementary DNA (cDNA), or ribonucleic acid (RNA).

IMMUNOCONJUGATES

Immunoconjugates (having whole antibody or Ig fusion proteins) may be constructed using a wide variety of chemotherapeutic agents such as folic acid and anthracyclines (Peterson et al., "Transport And Storage Of Anthracyclines In Experimental Systems And Human Leukemia", in Anthracycline Antibiotics In Cancer Therapy, Muggia et al. (Eds.), p. 132 (Martinus Nijhoff Publishers (1982); Smyth et al., "Specific Targeting of Chlorambucil to Tumors With the Use of Monoclonal Antibodies", J. Natl. Cancer Inst., 76:503-510 (1986)), including doxorubicin (DOX) (Yang and Reisfeld "Doxorubicin Conjugated with a Monoclonal Antibody Directed to a Human Melanoma-Associated Proteoglycan Suppresses Growth of Established Tumor xenografts in Nude Mice PNAS (USA)" 85:1189-1193 (1988)), Daunomycin (Arnon and Sela "In Vitro and in vivo Efficacy of Conjugates of Daunomycin With Anti-Tumor Antibodies" Immunol. Rev., 65:5-27 (1982)), and morpholinodoxorubicin (Mueller et al., "Antibody Conjugates With Morpholinodoxorubicin and Acid-Cleavable Linkers", Bioconjugate Chem., 1:325-330 (1990)).

BR96 has been conjugated to doxorubicin and has been shown to be effective in therapy of certain cancers or carcinomas (Trail, P.A., Willner, D., Lasch, S.J., Henderson, A.J., Casazza, A.M., Firestone, R.A., Hellström, I., and Hellström, K.E. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science, 261:212-215, 1993).

In accordance with the practice of the invention, structurally altered BR96 can be used in forms including unreduced IgG, reduced structurally altered IgG, and fusion proteins (PCT Application No. 95/305444, published March 6, 1996).

Suitable therapeutic agents for use in making the immunoconjugate includes Pseudomonas exotoxin A (PE) in either the native PE or LysPE40 form. LysPE40 is a truncated form containing a genetically modified amino terminus that includes a lysine residue for conjugation purposes. Doxorubicin is also a suitable therapeutic agent.

Additional examples of therapeutic agents include, but are not limited to, antimetabolites, alkylating agents, anthracyclines, antibiotics, and anti-mitotic agents.

Antimetabolites include methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine.

Alkylating agents include mechlorethamine, thiotepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin.

Anthracyclines include daunorubicin (formerly daunomycin) and doxorubicin (also referred to herein as adriamycin). Additional examples include mitozantrone and bisantrene.

Antibiotics include dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC).

Antimitotic agents include vincristine and vinblastine (which are commonly referred to as vinca alkaloids).

Other cytotoxic agents include procarbazine, hydroxyurea, asparaginase, corticosteroids, mytotane (O,P'-(DDD)), interferons.

Further examples of cytotoxic agents include, but are not limited to, ricin, bryodin, gelonin, supporin, doxorubicin, taxol, cytochalasin B, gramicidin D, ethidium bromide, etoposide, tenoposide, colchicine, dihydroxy anthracin dione, 1-dehydrotestosterone, and glucocorticoid.

Clearly analogs and homologs of such therapeutic and cytotoxic agents are encompassed by the present invention. For example, the chemotherapuetic agent aminopterin has a correlative improved analog namely methotrexate.

Further, the improved analog of doxorubicin is an Fe-chelate. Also, the improved analog for 1-methylnitrosourea is lomustine. Further, the improved analog of vinblastine is vincristine. Also, the improved analog of mechlorethamine is cyclophosphamide.

METHODS FOR MAKING MOLECULES OF THE INVENTION

There are multiple approaches to making site specific mutations in the CH_2 domain of an immunoglobulin molecule. One approach entails PCR amplification of the CH_2 domain with the mutations followed by homologous recombination of the mutated CH_2 into the vector containing the desired immunoglobulin, e.g., hBR96-2. For example, hBR96-2B and hBR96-2D have been made by this method.

Another approach would be to introduce mutations by site-directed mutagenesis of single-stranded DNA. For example, vector pD17-hG1b, which contains only the constant region of IgG1 and not the V domain of hBR96, has the fl origin of replication. This gives the vector the properties of a phagemid and site-directed mutagenesis experiments can be performed according to the methods of Kunkel, et al. (Kunkel, T.A., J.D. Roberts, and R.A. Zakour, 1987 Methods Enzymol. 154:367-383) as provided in the Bio-Rad Muta-Gene® phagemid *in vitro* mutagenesis kit, version 2. For example, hBR96-2B, -C, -D, -E, -F, -G, and -H were made by this method.

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the scope of this invention in any manner.

EXAMPLE 1

The following standard ELISA protocol was used.

Materials: Immulon2 96 well plates and Genetic Systems Specimen Diluent Concentrate (10x); antibody conjugate was Goat Anti Human Kappa-HRP Mouse Adsorbed, Southern Biotech. at 1:10,000 in Genetic Systems Conjugate Diluent (1x); Genetic Systems EIA Chromogen Reagent (TMB) (1:100); Genetic Systems EIA Buffered Substrate (1x); primary antibody or antigen were AffiniPure $F(ab')_2$ Fragment Goat Anti Human IgG Fc Fragment specific (Jackson Immuno Research), Goat Anti Human Kappa-UNLB (Southern Biotechnology Associates), Le^y-HSA (Alberta Research Council).

Methods: Dilute primary antibody or antigen to 1.0 μg/ml in 0.05M Carb/Bicarb buffer. Add 100μl of the diluted solution per well in Immulon 2 plates. Seal plates and incubate O.N. at 4°C.

Block plates by flicking them and blotting on paper towels. Add 200μl/well of Genetic Systems, Specimen Diluent Concentrate (1x). Incubate at least 1 hour at room temperature and then dump the contents of the plates. Wash the plates 3x in saline/Tween. Blot to dry. Allow the plates to dry at R.T. (45 min. to 1 hour). Seal and store the plates at 4°C.

Test samples as follows. Dilute samples and standards in Specimen Diluent at 1:10. Perform serial dilutions in separate round bottom plates. Transfer 100μl/well of final dilutions to antigen coated assay plates; then incubate O.N. at 4°C. Wash plates 3x with saline/Tween.

For conjugation add 100 μl/well of antibody-HRP conjugate in Genetic Systems Conjugate Diluent (1x). Incubate plates at Room Temp. for 60 min. Wash plates 3x in saline/Tween.

Add 100 μl/well of Genetic Systems EIA Chromogen Reagent (TMB) 1:100 in EIA Buffered Substrate (1x). Incubate at R.T. for 15 min. and stop with 1N H_2SO_4 100 μl/well. Read plate at 450/630nm in EIA plate reader.

EXAMPLE 2

Construction of CH_2 deleted BR96 molecules

Strategy for Deleting CH_2 Domains: To construct CH_2 deleted BR96 molecules, the hinge, CH_2 and CH_3 domains were removed from chimeric BR96 and humanized

BR9696-2 IgG1 molecules by an Eco47-III restriction digestion in non-coding regions. The hinge and CH_3 domains were amplified by polymerase chain reaction (PCR) from a human IgG1 (pNγ1.14) molecule lacking the CH_2 domain. Two oligonucleotides (Sense 49mer, Antisense 50mer) homologous to the sequences of IgG1 constant region at both sides preserving E.co47-III sites were synthesized. The amplified hinge and CH_3 domain PCR fragments were added into Eco47-III sites on BR96 IgG1 molecules by in vivo homologous recombination (P. Bubeck et al., Nucleic Acid Research (1993) 21:3601-3602). The new BR96 IgG1 molecules were verified by restriction mapping and sequencing.

A sewing PCR strategy was used for the construction of CH_2 deleted human IgG1 (pNγ1.14) (Robert M. Horton, et al. (1990) Biotech 8 (5)P, 528).

The CH_1 domain was amplified as a 580 bp fragment with a sense oligonucleotide (5' TGG CAC CGA **AAG CTT** TCT GGG GCA GGC CAG GCC TGA 3') (primer A) and an antisense oligonucleotide (5' **TCC GAG CAT GTT GGT ACC CAC GTG GTG GTC GAC** GCT GAG CCT GGC TTC GAG CAG ACA 3') (primer B) from a linearized human IgG1 constant region vector (pNγ1.7). The PCR fragment extends from the 5' end of the Hind-III site (in bold) through the Cel-II, Sal-I, Dra-III, Kpn-I, 6 bp nucleotide spacer and Mro-I sites (in bold) at the 3' end of the CH_1 domain.

The CH_3 domain was then partially amplified (to the Xba-I site) with a sense primer (5' **GTC GAC CAC CAC GTG GGT ACC AAC ATG TCC GGA** GCC ACA TGG ACA GAG GCC GGC T 3') (primer C) and an antisense primer (5' CTG GTT CTT GTT CAT CTC CTC **TCT AGA** TGG 3') (primer D) from a linearized human IgG1 constant region vector (pNγ1.7). A PCR fragment (about 150 bp) with Sal-I, Dra-III, Kpn-I, 6 nucleotide spacer and Mro-I sites (in bold) on its 5' end, extends only through the Xba-1 site (in bold) within the CH_3 domain.

The CH_1 and CH_3 partial PCR fragments were combined in a PCR without any primer. The reaction was run through two full cycles of denaturation and re-annealing to allow the fragments to combine at the homologous region at the 3' ends. Primers A and D (described above) were added to the reaction and the PCR cycle was completed. The polymerase extends the DNA with primer A and primer D, yielding a full- length (660 bp) PCR fragment. The newly extended PCR fragment is arranged from the 5' end to the 3' end in the following order: Hind-III - CH_1 - Cel-II - Sal-I - Dra-III - Kpn-I - 6 bp spacer - Mro-I - CH_3 partial - Xba-1.

The combined PCR fragment, with the CH_1 and partial CH_3 domains, was then cloned by a blunt end ligation into a Sma-I site on a pEMBL18 vector and the sequence was confirmed by dideoxy sequencing (Sanger et al. (1977) PNAS (USA) 74:5463-5466).

To transfer the CH_1 and partial CH_3 into a mammalian expression vector, both the pEMBL18 and pNγ1.7 vectors were digested with Hind-III and Xba-I. The Hind-III and Xba-I fragment was ligated into the same sites on a linearized pNγ1.7 vector. The new construct, with CH_1 and a full CH_3 domain, was designated the pNγ1.10 vector.

The hinge fragment was amplified from a Hind-III digested pNγ1.7 vector with the primers designed to flank the hinge exon with a Sal-I and a Dra-III cloning site at each end. These sites also exist between the CH_1 and CH_3 domains of the pNγ1.10 construct. The sense oligonucleotide (5' ACC ATG **GTC GAC** CTC AGA CCT GCC AAG AGC CAT ATC 3') with a 6 bp spacer and a Sal-I cloning site (in bold) and the antisense oligonucleotide (5' CAT GGT **CAC GTG** GTG TGT CCC TGG ATG CAG GCT ACT CTA G 3') with a 6 bp spacer and a Dra-III cloning site (in bold) were used for the amplication of the hinge fragment (250 bp).

The hinge region PCR fragment was cloned into a Sma-I site on pEMBL18 by blunt end ligation. Both the pEMBL18 with the hinge domain and the pNγ1.10 with the CH_2 and CH_3 domains were digested with Sal-1 and Dra-III. The digested hinge fragment was cloned into the Sal-1 and Dra-III linearized sites on the pNγ1.10 vector. The new construct, now carrying the CH_1, hinge and CH_3 domains, was designated pNγ1.11.

To make the final CH_2 deleted human IgG1 construct, both the pNγ1.11 construct and pNγ1.11 vector were digested with BamH1 and HindIII. A fragment containing the CH_1, hinge and CH_3 domains was cloned into the linearized pNγ1.11 vector. The new constant region IgG1 construct lacks the CH_2 domain and is designated pNγ1.14 (Figure 11).

For digestion of BR96 IgG1 with Eco47-III, a restriction fragment with hinge, CH_2 and CH_3 domains was identified on the constant region sequence of BR96 IgG1 vector in both chimeric and humanized molecules. The 5' end of this fragment lies inside the intron between CH_1 and hinge and the 3' end is located inside the CH_3 intron of the BR96 IgG1 molecule. The hinge, CH_2 and CH_3 domains (1.368 kb fragment) were removed from BR96 IgG1 molecules by Eco47-III restriction digestion. The Eco47-III is a blunt end cutter. The BR96 IgG1 DNA digested with this enzyme does not require any pretreatment before cloning. Figure 12 is a diagrammatic representation of the pD17-hBR96-2 vector showing the Eco47-III sites used in cloning.

The CH_2 deleted BR96 IgG1 was then constructed as follows. The hinge and CH_3 domains were amplified from a CH_2 deleted L6 IgG1 (pNγ1.14) construct with a sense oligonucleotide (5'
CAGGGAGGGAGGGTGTCTGCTGGAAGCCAGGCTC**AGCGCT**GACCTCAG

A 3') homologous to the constant region sequence of IgG1 at the 5' end of the Eco47-III site (in bold) and an antisense oligonucleotide (5'GGAAAGAACCATCACAGTCTCGCAGGGG CCCAGGGCA**GCGCT**GGGTGCTT 3') homologous to the constant region sequence of IgG1 at the 3' end of the Eco47-III site (in bold). The Eco47-III site at the 3' end of the pNγ1.14 construct is modified in the cloning process. The Eco47-III site is thus introduced into an antisense primer and used in amplification of the hinge and CH_3 domains.

The pD17-BR96 IgG1 vector was digested with Eco47-III and the hinge, CH_2 and CH_3 domains were removed. The linearized pD17-BR96 IgG1 vector was mixed with equimolar amounts of hinge and CH_3 PCR fragments. Cotransformation of the PCR fragment with linearized DNA into E.coli DH5a competent cells resulted in a recombinant molecule, mediated by homologous recombination in bacteria. This construct lacks the CH_2 domain of BR96 IgG1 molecules, and is designated pD17-BR96-dCH2 (Figure 13).

1.9 grams of CH_2-deleted chimeric BR96 was obtained as raw material from 89L of culture supernatant.

EXAMPLE 3

Toxicity, localization and clearance of CH_2-deleted chimeric BR96 was tested in vivo as follows.

Three dogs received 400 mg/m^2 of cBR96-A, the CH_2 deletion mutant of chimeric BR96, and two received chimeric BR96. Both molecules had been mildly reduced and alkylated. This is required to prevent dimerization of the deletion mutant into a tetravalent form. Both control dogs experienced the typical GI toxicity and none of

the three receiving the mutant displayed any toxicity. The control dogs and two of the test dogs were sacrificed at 1 hr to obtain duodenal tissue to measure antibody localization. Both control dogs had grossly visible GI pathology, and the test dogs had normal appearing GI tissue. The third dog has continued to show no signs of toxicity.

Results: A significant amount of localization of the CH_2 deleted cBR96 (cBR96-A) occurred to the GI tract in dogs treated with 400 mg/m^2, although the intact chiBR96 localized slightly better. The levels of localization indicate that roughly equivalent amounts of intact and CH_2 deleted cBR96 was delivered to the GI tract in these dogs.

Table 5. Localization of cBR96 to GI tissue.

Group	Animal	Specific Localization	mean
cBR96	#271	155	135
	#272	114	
cBR96-A	#273	126	89
	#274	52	

Using the mean level of specific localization, an amount of cBR96-A equivalent to at least 66% of the amount of cBR96 was delivered to the target organ of toxicity, the duodenum. Based on the dose ranging done with cBR96 in dogs (some clinical signs of toxicity seen at doses of 10 mg/m^2), even if this difference is real, it could

not explain the difference between significant toxicity and no toxicity, evaluation to date indicated that dogs treated with cBR96-A had no toxicity, pending microscopic histopathologic examination. This evaluation was based on analysis of 2 frozen blocks per dog and 2 sections per block. Replicates were quite good. We also ran historical frozen tissues from dogs treated with native cBR96 or F(ab)2/BR96 and the levels of localization for those tissues were 110 and 0, respectively, consistent with our previous data.

Assuming that there is no toxicity at marginally higher (2X) doses of cBR96-A, these data indicate that the CH_2 domain is associated with the induction of acute gastroenteropathy, and that the removal of this domain prevents the induction of gastroenteropathy mediated by BR96.

This study confirms the results showing that F(ab')2 is not toxic in the dog model and that the toxicity is mediated by the constant region. The CH_2 deletion mutant is a candidate for targeting agents clinically. Because of the very long half-life of chimeric BR96, some decrease in the mutant's half-life should be acceptable.

Figure 1 shows the measurement of the clearance of the cBR96-A in high Le^Y expressing dogs. The study used chimeric versus constant region mutant of cBR96-2.

CBR96-2 did clear faster than the chimeric BR96. The localization of cBR96-A to the gastrointestinal epithelium is not significantly affected by this more rapid clearance. More than enough of the cBR96-A localized to have caused toxicity.

Discussion: The constant region of chimeric IgG is responsible for the GI toxicity seen in clinical trials, e.g. with chiBR96-dox. The GI toxicity seen in the dog model is very similar to the clinical toxicity. Both in man and dog, administration of the

unconjugated antibody mediates an acute GI toxicity characterized by rapid onset of vomiting, often with blood.

In man the bleeding is limited to the fundus of the stomach, causing erosion of the superficial mucosa of the stomach. Although the pathology of the wound is limited and resolves, the extreme nature of the nausea and vomiting, unrelieved by anti-emetics, defines it as the dose-limiting toxicity.

This toxicity is mediated in man and dog by the antibody molecule alone. At higher doses of the antibody-dox conjugate, additional toxicity is seen in the dog model, probably due to doxorubicin. Although the intact IgG of BR96 causes toxicity in dog and man, the F(ab')2 molecule (divalent and lacking only in the constant region) is not toxic in dogs. This finding has motivated our attempts at high levels, and improves the affinity and specificity of BR96 for tumor antigen.

The CH_2 domain is known to mediate complement and FcR binding. It was not known that structural alteration of the CH_2 domain would result in immunoglobulin-induced toxicity inhibition.

Toxicology study of hBR96-2B

The toxicology study of hBR96-2B in high Lewis Y expressor dogs (n=2) showed that a dose of 400 mg/m^2 did not cause hematemesis nor bloody stools, in contrast to BR96 which consistently causes one or both signs. A dog sacrificed at 24 hrs had normal gross appearance of the GI tract, again in marked contrast to chimeric BR96 which causes hemorrhagic lesions and mucosal erosions.

EXAMPLE 4

The polymerase chain reaction (PCR) is a widely used and versatile technique for the amplification and subsequent modification of immunoglobulin genes. The rapidity and accuracy with which antibody genes can be modified in vitro has produced an assortment of novel antibody genes can be modified in vitro has produced an assortment of novel antibodies. For example, PCR methods have been used for engineering antibodies with increased affinity to antigen, for "humanizing" antibodies, and for modulating effector function (Marks, J.D., A.D. Griffiths, M. Malmqvist, T. Clackson, J.M. Bye and G. Winter. 1992. Bypassing immunization: high affinity human antibodies by chain shuffling. Bio/Technology 10:779-783; Rosok, M.J., D.E. Yelton, L.J. Harris, J. Bajorath, K.-E. Hellstrom, I. Hellstrom, G.A. Cruz, K. Kristensson, H. Lin, W.D. Huse and S.M. Glaser. 1996. A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. J. Biol. Chem. 271:22611-22618; Morgan, A.N., D. Jones, A.M. Nesbitt, L. Chaplin, M.W. Bodmer and S. Emtage. 1995. The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for Clq, FcγRI and FcγRIII binding. Immunology. 86:319-324).

As part of a more comprehensive study, we desired to introduce various site specific mutations in the CH_2 constant domain of human IgG_1. Six specific amino acid residues distributed throughout the CH2 domain previously identified to play a role in immune effector function were marked as targets for mutagenesis (Morgan, A.N., D. Jones, A.M. Nesbitt, L. Chaplin, M.W. Bodmer and S. Emtage. 1995. The N-terminal end of the CH2 domain of chimeric human IgG1 anti-HLA-DR is necessary for Clq, FcγRI and FcγRIII binding. Immunology. 86:319-324; Duncan, A.R. and G. Winter. 1988. The binding site for C1q on IgG. Nature 332:738-740; Tao, M.-H., R.I.F. Smith and S.L. Morrison. 1993. Structural features of human immunoglobulin G that determine isotype-specific differences in complement

activation. J.Exp.Med. 178:661-667). five of the six residues were grouped into two clusters-one cluster consisting of two residues, two amino acids apart (Location 1, or L1); and a second cluster consisting of three residues spanning a sequence of five amino acids (L2). The remaining amino acid position (L3) made for the total of six residues. We were interested in constructing a panel of mutant CH_2 domain IgGs consisting of each L mutation by itself as well as in combination with other L mutants (e.g., L1; L1; and L2; L1, L2 and L3; etc.).

Various *in vitro* methods have been described where PCR is used to simultaneously introduce distally located site-specific mutations within a gene sequence (Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension. Gene 77:51-59; Ge, L. and P. Rudolpf. 1996. Simultaneous introduction of multiple mutations using overlap extention PCR. BioTechniques 22:28-30). Alternatively, an *in vivo* procedure termed recombination PCR (RPCR) has also successfully been used for rapidly and efficiently generating distally located site-specific mutations (Jones, D.H. and S.C. Winistorfer. 1993. Use of polymerase chain reaction for making recombinant constructs. p.241-250. In B.A. White (Ed.), Methods in Molecular Biology, Vol. 15. Humana Press Inc., Totowa, NJ, Jones, D.H. And B.H. Howard. 1991. A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction. BioTechniques 10:62-66). RPCR uses *E. Coli's* recombination machinery to generate intact circular recombinant plasmids from a transfected mixture of linear PCR-generated product and linearized vector. *In vivo* recombination is mediated through the joining of nucleotide sequences designed into the 5' ends of both PCR primers that are homologous to DNA sequences encoded by the vector. In this report we describe an extension of the RPCR procedure for simultaneously introducing complex combinations of mutations into an antibody CH_2 domain.

Humanized BR96 variable region heavy and light chain genes, previously cloned and co-expressed as an assembled active Fab fragment in an M13 phage expression vector, provided the starting material (Rosok, M.J., D.E. Yelton, L.J. Harris, J. Bajorath, K.-E. Hellstrom, I. Hellstrom, G.A. Cruz, K. Kristensson, H. Lin, W.D. Huse and S.M. Glaser. 1996. A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. J. Biol. Chem. 271:22611-22618). The heavy and light chain V genes were amplified by PCR from a single-stranded M13 DNA template and subcloned by *in vivo* recombination (Jones, D.H. And B.H. Howard. 1991. A rapid method for recombination and site-specific mutagenesis by placing homologous ends on DNA using polymerase chain reaction. BioTechniques 10:62-66) into vectors pD17-hG1a and pD16-hCκ, to form pBR96-hG1a and pBR96-hCκ respectively. pD17-hG1a and pD16-hCκ are eukaryotic immunoglobulin expression vectors derived from pcDNA3 (Invitrogen, San Diego, CA). The plasmid pBR96-hG1a was further modified by site-directed mutagenesis to introduce two Eco47-III restriction sites flanking the immunoglobulin hinge-CH_2-CH_3 domains using standard procedures. The recipient vector was then prepared by digesting pBR96-hG1a with Eco47-III, isolating the vector backbone by agarose gel electrophoresis followed by extracting the vector DNA from the excised gel slice using the Qiagen Gel Extraction kit (Qiagen, Chatsworth, CA).

The strategy for introducing multiple mutations within the immunoglobulin CH_2 gene, shown in Figure 24, relies on the *in vivo* homologous recombination of several independently amplified PCR products with each other as well as with the pBR96-hG1a vector DNA. For introducing mutations at two distal locations two PCR products are synthesized (Figure 24B). One end of each PCR product is for recombining with an homologous end of the linear vector, and the other end, encoding the mutation(s) of interest, is for recombining with the neighboring PCR product. As shown in Figure 24B, additional distally-located mutations can be introduced into a target sequence by increasing the number of PCR products

proportionately. The recombination of neighboring PCR products always occurs across the regions containing the desired mutations, therefore the oligonucleotide primers encoding these ends (e.g., A1, A2) contain complementary mutant residues. The mutagenic PCR primers contain at least 15 nucleotides of wild-type sequence flanking each side of the mutant residues for either priming the polymerization reaction or mediating recombination. Two 49-nucleotide long PCR sense and anti-sense primers (Rs and Ra) contain sequences for recombining with the end regions of the Eco47-III digested pBR96-hG1a vector.

Each L mutation was amplified in a separate PCR reaction. The reaction conditions were 250 ng intact pBR96-hG1a DNA template, 10 ul of 1X *Pfu* buffer (Stratagene, Inc. San Diego, CA), 10 nmol dNTPs, 200ng each of the appropriate PCR primers, 10% dimethysulfoxide (ATCC, Rockville, MD) and 2.5 units cloned *Pfu* DNA polymerase in a 100ul reaction volume. Samples were first denatured at 95° C for 5 min, cooled to 45°C for 5 min, and extended at 72°C for 1 min followed by 25 cycles of denaturation at 94°C for 45 sec, annealing at 45°C for 45 sec, extension at 72°C for 1 min/kb, followed by a final extension at 72°C for 7 min in a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT). The amplified products were purified from a 1% agarose gel, extracted with Qiagen Gel Extraction kit and the recovered DNA quantitated. 50 ng of each PCR product was mixed with 25 ng of the Eco47-III digested pBR96-hG1a vector, transfected into Max competent E. coli DH5α according to the manufacturer's procedure (GIBCO BRL/Life Technologies, Gaithersburg, MD), and the entire transfection reaction plated onto selective LB agar plates containing 100 ug/ml ampicillin.

The results of several cloning experiments are summarized in the Table that follows. Typically the transformations produced from 80 to 200 bacterial colonies. Individual colonies were selected and grown overnight in 2 ml liquid cultures for isolation of miniprep plasmid DNA (Qiagen) and analysis by Eco47-III restriction

endonuclease mapping. Among 24 independent transformants analyzed from triple homologous recombination events (two PCR products plus vector) 11 clones contained the predicted 1.4 kpb DNA insert.

Figure 25 shows a sample diagnostic restriction analysis of DNA prepared from clones derived from quadruple homologous recombination events (three PCR products plus vector). Additional sampling of clones resulting from quadruple recombination yielded a cloning efficiency of 29% (7 clones containing inserts/24 clones sampled). At this point, due to the small sampling sizes, we do not know whether the differences in the cloning efficiencies observed between the triple and quadruple recombination events are meaningful.

To evaluate the expression of Le^{y} -binding activity of the CH_2 mutant IgGs, miniprep DNAs from 6 clones derived from the triple recombination reaction and 6 clones derived from the quadruple recombination reaction exhibiting the predicted diagnostic Eco47-III restriction patterns were isolated, mixed with pBR96- hCκ DNA and used to co-transfect COS7 cells. 48 hour spent supernatants from 3 ml cultures were assayed for total IgG production and for Le^{y} binding activity by enzyme-linked immunosorbent assay (EIA) as described (Yelton, D.E., M.J. Rosok, G.A. Cruz, W.L. Cosand, J. Bajorath, I. Hellstom, K.-E. Hellstorm, W.D. Huse and S.M. Glaser. 1995. Affinity maturation of the BR96 anti-carcinoma antibody by codon-based mutagenesis. J.Immunol. 155:1994-2004). All twelve cultures were found to secrete approximately 2-3 ug/ml Le^{y} -reactive IgG. The spectrum of Le^{y} binding activities were all similar to that of native humanized BR96 IgG indicating that the homologously recombined antibodies did not acquire any gross mutations that could affect antigen binding. To confirm that the desired CH_2 mutations had been incorporated, and to evaluate the recombined genes for misincorporated nucleotides, four of the clones producing functional antibody were sequenced using Sequenase Version 2 DNA Sequencing Kit (United States Biochemical). One clone

was found to contain a single nucleotide change within the forward PCR primer used for mediating recombination with vector DNA. We are uncertain whether this error occurred during chemical synthesis of the oligonucleotide primer or is a result of misincorporation during the PCR reaction, despite the fact that we used a thermostable polymerase with proofreading activity.

A RPCR procedure for homologously recombining up to three separate PCR-generated mutated antibody sequence products into a eukaryotic expression vector for the rapid construction of engineered IgG molecules is described herein. The advantage of this approach is the ability to simultaneously introduce multiple distally-located mutations with PCR products synthesized by a single round of PCR. Recombinant DNAs are produced with a reasonably high cloning efficiency and fidelity of correct nucleotide sequences. The ability to efficiently rejoin several distinct PCR products should permit combinatorial strategies for constructing complexly mutated protein domains as well as broadening the number and location of desired mutations.

Analysis of transformants generated by multiple-fragment RPCR.

Mutant IgGs Constructed	PCR Fragments in reaction	HR[a] events	Colonies Analyzed	Cloning Efficiency[b]
2	2	triple	24	45%
2	3	quadruple	24	33%

[a]HR-homologous recombination

[b]Cloning efficiency (number of clones containing 1.4kbp insert/total number of colonies

EXAMPLE 5

This example provides two methods for introducing site specific mutations into the CH2 domain of human IgG1 constant region containing vectors.

One method involves PCR amplification of a segment or segments of the constant region, wherein mutations are introduced using appropriately constructed oligonucleotides. The vector receiving the fragment(s) is digested with a restriction enzyme to linearize the vector. PCR amplification primers are designed so that the 5' ends of the PCR fragments can hybridize to the DNA sequence of the vectors. If more than one PCR fragment is amplified, then common sequences to the two fragments are introduced by oligonucleotides. Bacteria are transfected with the PCR fragments and with the digested vector. The fragments and vector can recombine by homologous recombination using the bacteria's recombination machinery. Bacterial colonies are selected and the DNA is analyzed by size and restriction map as a preliminary determination that the vector and fragment(s) recombined correctly. Correct insertion of fragments with the mutations is confirmed by dideoxynucleotide sequence analysis. DNA is then introduced into mammalian cells as described for the CH2 deleted antibody, and the expressed antibody analyzed for binding and functional activity.

By way of example, mutations Leu to Ala at residue 235 in CH2 and Gly to Ala at residue 237 were introduced by the procedure disclosed in Example 4. The heavy chain vector used for this procedure was pD17-hG1a, similar to pD17-BR96 vector described herein except that humanized V regions (Rosok, M.J., D.E. Yelton, L.J. Harris, J. Bajorath, K-E. Hellstrom, I, Hellstrom, G.A. Cruz, K. Kristensson, H. Lin, W.D. Huse, and S.M. Glaser, 1996. J. Biol. Chem 271 37:22611-22618) with three affinity mutations (H1, H2, and H3 mutations) were substituted.

pBR96-hG1a contains two Eco47-III restriction sites flanking the Ig hinge-CH2-CH3 domains. The recipient vector was prepared by (1) digesting pBR96-hG1a with *Eco*47-III, (2) isolating the vector by agarose gel electrophoresis, and (3) extracting the vector DNA from the excised gel slice using the Qiagen Gel Extraction kit (Qiagen, Chatsworth, CA). To introduce mutations at a single location, such as for positions 235 and 237, two PCR products were synthesized.

To introduce two distally located mutations, such as for mutant F (also referred to herein as hBR96-2F) with mutations at 235, 237, 331, requires 3 PCR products. The recombination of neighboring PCR products occurs across the regions containing the desired mutations, therefore the oligonucleotide primers encoding these ends contain complementary mutant residues. The mutagenic PCR primers contain at least 15 nucleotides of wild-type sequence flanking each side of the mutant residues for either priming the polymerization reaction or mediating recombination. Two 49-nucleotide long PCR sense and anti-sense primers containing sequences for recombining with the end regions of the *Eco*47-III digested pBR96-hG1a vector.

PCR amplification used 250 ng intact pBR96-hG1a DNA template, 10 μl of 10X *Pfu* buffer (Stratagene, Inc., San Diego, CA), 10 nmol dNTPs, 200 ng each of the appropriate PCR primers, 10% dimethylsulfoxide (ATCC, Rockville, MD) and 2.5 units cloned *Pfu* DNA polymerase (Stratagen, Inc. San Diego, CA) in 100 μl reaction. Samples were denatured at 95°C for 5 min, annealed at 45°C for 5 min, and extended at 72°C for 1 min followed by 25 cycles of denaturation at 94°C for 45 sec, annealing at 45°C for 45 sec, extension at 72°C for 1 min/kb, and a final extension at 72°C for 7 min. The amplified products were purified from a 1% agarose gel, extracted with the Qiagen Gel Extraction kit and quantitated. 50 mg of each PCR product was mixed with 25 ng of the Eco47-III digested pBR96-hG1a vector and transfected in E.coli MAX Efficiency DH5α™ according to the

manufacturer's instructions (GIBCO BRL/Life Technologies, Gaithersburg, MD). The entire transfection reaction was plated onto LB agar plated containing 100 μg/ml ampicillin.

Bacterial colonies were selected and grown overnight at 37° C in 2 ml liquid cultures. DNA was isolated and analyzed by Eco47-III restriction endonuclease mapping. Clones with the correct size insert were sequenced (Sequenase Version 2, U.S. Biochemical Corp., Cleveland, OH).

The second method for introducing site specific mutations into the CH_2 domain of human IgG1 involved the method of Kunkel (1987 Methods Enzymology, supra). For this procedure pD17-hG1b DNA with the F1 origin of replication was introduced into electrocompetent E. coli CJ236 dut-ung- (Bio-Rad Laboratories, Hercules, CA) by electroporation according to manufacturer's instructions. PD17-hG1b is a vector having a constant region but no variable region. The F1 ori site allows treatment of this vector as a phagemid.

Bacteria containing the plasmid were selected by ampicillin resistance. Single stranded uridinylated DNA was prepared using the Muta-Gene Phagemid In Vitro Mutagenesis Version 2 protocol (Bio-Rad). Mutations were introduced by site-directed mutagenesis with the appropriate antisense oligonucleotide. For molecules with mutations at more than one location, mutations were introduced by either of the two methods discussed above. One method would be to (1) prepare one mutant, for example, mutant 2C (also referred to herein as BR96-2C) with the mutations at residues 318, 320, 322, (2) isolate ssDNA, and (3) introduce a second mutation set with the appropriate anti-sense oligonucleotide. The second method would be to anneal two antisense oligonucleotides with the same uridinylated ssDNA and screen for mutants with both sets of changes. Mutant 2H (hBR96-2H) was also prepared by a combination of thse methods.

The V region of humanized BR96-2 heavy chain was introduced by the homologous recombination method described above in pD17-hJm14.H1. The pD17-hJm14.H1 plasmid contains the BR96 humanized variable region with the H1/H2/H3 mutations and the plasmid was used to transfect mutant sequences into mammalian cells. The pD17G1b vector containing the Fc mutation(s) was digested with NheI for 3 hr at 37° C and the DNA isolated by methods described above. Insertion of the V region into the vector was determined by size and restriction enzyme mapping and confirmed by sequence analysis.

Transient expression of whole antibodies was performed by transfection of COS cells. For production of antibody, stable transfections of CHO cells were performed (see description of deleted CH2 mutant). All mutants were purified from CHO culture supernatants by protein A chromatography.

The oligonucleotide primers homologous to the vector and used to introduce the constant regions mutations were as follows:

Oligonucleotides homologous to vector sequences:

Sens(sense)CH2 E47-3-5: CAG GGA GGG AGG GTG TCT GCT GGA AGC CAG GCT CAG CGC TGA CCT CAGA

D CH2 E47-3 A (antisense): GGA AAG AAC CAT CAC AGT CTC GCA GGG GCC CAG GGC AGC GCT GGG TGC TT

Oligonucleotides to mutate Leu235 to Ala and Gly237 to Ala (underlined sequences show sites of mutation):

Antisense CH2 L235-G237/aa: GAA GAG GAA GAC TGA CGG TGC CCC CGC GAG TTC AGG TGC TGA GG

SensCH2 L235-G237/AA: CCT CAG CAC CTG AAC TCG CGG GGG CAC CGT CAG TCT TCC TCT TC

Oligonucleotides to mutate Glu318, Lys320, Lys322 to Ser

Antis(antisense)CH2 EKK/SSS-2: CTG GGA GGG CTT TGT TGG AGA CCG AGC ACG AGT ACG ACT TGC CAT TCA GCC

Oligonucleotides to mutate Pro331 to Ala:

Antis CH2 P331/A/3: GAT GGT TTT CTC GAT GGC GGC TGG GAG GGC

Sense CH2 P33/A: GCC CTC CCA GCC GCC ATC GAG AAA ACC ATC

Alternative antisense oligo to introduce Ala at 331 by site-directed mutation:

CH2P331A: GAT GGT TTT CTC GAT AGC GGC TGG GAG GGC TTT G

Oligonucleotides to mutate Glu318 to Ser, Lys320 to Ser, Lys322 to Ser, and Pro331 to Ala:

Antis CH2 EKKP/SSA-6: GAT GGT TTT CTC GAT GGC GGC TGG GAG GGC TTT GTT GGA GAC CGA GCA CGA GTA CGA CTT GCC ATT CAG CCA GTC CTG GTG

Sense CH2 EKKP/SSA-6: CAC CAG GAC TGG CTG AAT GGC AAG TCG TAC TCG TGC TCG GTC TCC AAC AAA GCC CTC CCA GCC GCC ATC GAG AAA ACC ATC

In vitro Assays of the Mutants

Results of the CDC demonstrate that mutant hBR96-2B has approximately 10 fold less activity than the control hBR96-1 (two affinity mutations, one in H2 and one in H3, refer to previous patent (Figure 20)). The mutants that have the least ability to kill cells in the presence of complement is hBR96-2C with the triple mutations at positions 318, 320, and 322 and the hBR96-2H mutant (least cytotoxic antibodies in the panel) which contains all six mutations at the three different locations. ADCC activity was most affected by the CH2 deleted hBR96-2 molecule (Figure 21).

hBR96-2B and -2H lost between 100 and 1000 fold activity to kill in the presence of effector cells. In the ADCC assay the hBR96-2B molecule also lost approximately 10 fold activity (Figure 21).

Figures 26-28 provide the amino acid sequences for the heavy chain variable region for both chimeric and humanized BR96 having the H1, H2, and H3 mutations. The amino acid sequence for the light chain variable region is known and methods for generating it are found in PCT Application No. 95/305444. Additionally provided is the amino acid sequence for the IgG1 constant region. Mutations in the constant region are marked.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Bristol-Myers Squibb Co.

(ii) TITLE OF THE INVENTION:
A METHOD FOR INHIBITING
IMMUNOGLOBULIN-INDUCED TOXICITY FROM THE USE OF
IMMUNOGLOBULINS IN THERAPY AND IN VIVO DIAGNOSIS

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant & Gould
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(C) CITY: Los Angeles
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 90025

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US97/____________.
(B) FILING DATE: 01-AUG-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/023,033
(B) FILING DATE: 02-AUG-1996

(viii) ATTORNEY/AGENT INFORMATION:
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(A) TELEPHONE: 310-445-1140
(B) TELEFAX: 310-445-9031
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGCACCGAA AGCTTTCTGG GGCAGGCCAG GCCTGA 36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCCGGACATG TTGGTACCCA CGTGGTGGTC GACGCTGAGC CTGGCTTCGA GCAGACA 57

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCGACCACC ACGTGGGTAC CAACATGTCC GGAGCCACAT GGACAGAGGC CGGCT 55

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGGTTCTTG TTCATCTCCT CTCTAGATGG 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACCATGGTCG ACCTCAGACC TGCCAAGAGC CATATC 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATGGTCACG TGGTGTGTCC CTGGATGCAG GCTACTCTA 39

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGGGAGGGA GGGTGTCTGC TGGAAGCCAG GCTCAGCGCT GACCTCAGA 49

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAAAGAACC ATCACAGTCT CGCAGGGGCC CAGGGCAGCG CTGGGTGCTT 50

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8691 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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CCTTTTTTTT TAATTTTATT TTATTTTATT TTTGAGATGG AGTTTGGCGC CGATCTCCCG 120

```
ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAGTATC   180
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CAAGGCAAGG CTTGACCGAC AATTGCATGA AGAATCTGCT TAGGGTTAGG CGTTTTGCGC   300
TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT AGTTATTAAT   360
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TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA   480
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ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC   600
CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT   660
GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC   720
GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC   780
TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA   840
AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG   900
TCTATATAAG CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTACTGG CTTATCGAAA   960
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GTGAAGTGAA TCTGGTGGAG TCTGGGGGAG GCTTAGTGCA GCCTGGAGGG TCCCTGAAAG  1200
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GGAACTCAGG CGCCCTGACC AGCGGCGTGC ACACCTTCCC GGCTGTCCTA CAGTCCTCAG  1680
GACTCTACTC CCTCAGCAGC GTGGTCACCG TGCCCTCCAG CAGCTTGGGC ACCCAGACCT  1740
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CATCCCGGCT ATGCAGCCCC AGTCCAGGGC AGCAAGGCAG GCCCCGTCTG CCTCTTCACC  1920
CGGAGGCCTC TGCCCGCCCC ACTCATGCTC AGGGAGAGGG TCTTCTGGCT TTTTCCCCAG  1980
GCTCTGGGCA GGCACAGGCT AGGTGCCCCT AACCCAGGCC CTGCACACAA AGGGGCAGGT  2040
GCTGGGCTCA GACCTGCCAA GAGCCATATC CGGGAGGACC CTGCCCCTGA CCTAAGCCCA  2100
CCCCAAAGGC CAAACTCTCC ACTCCCTCAG CTCGGACACC TTCTCTCCTC CCAGATTCCA  2160
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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8327 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA 8100
CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG 8160
CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC 8220
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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8897 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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TCATTGTACA TAATAATGGC AACACCTATT TAGAATGGTA CCTGCAGAAA CCAGGCCAGT    240
CTCCACAGCT CCTGATCTAC AAAGTTTCCA ACCGATTTTC TGGGGTCCCA GACAGGTTCA    300
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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 8321 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 8897 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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What is claimed is:

1. A method for inhibiting immunoglobulin-induced toxicity resulting from immunoglobulin immunotherapy in a subject comprising administering an immunoglobulin molecule to the subject, the immunoglobulin molecule having a variable region and a constant region, the immunoglobulin molecule being modified prior to administration by structurally altering multiple toxicity associated domains in the constant region so that immunoglobulin-induced toxicity is inhibited.

2. A method for inhibiting immunoglobulin-induced toxicity resulting from immunoglobulin immunotherapy in a subject comprising administering a structurally altered antibody to the subject, the structurally altered antibody comprising a variable region and a constant region, multiple toxicity associated domains in the constant region being modified so as to render the constant region unable to mediate an ADCC response or activate complement thereby inhibiting immunoglobulin-induced toxicity resulting from immunotherapy.

3. A method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy in a subject comprising administering an Ig fusion protein to the subject, the Ig fusion protein having multiple structurally altered toxicity associated domains in the constant region.

4. A method for inhibiting immunoglobulin-induced toxicity resulting from immunotherapy in a subject comprising administering an Ig fusion protein to the subject, the Ig fusion protein comprising a modified constant region, the

modification being a structural alteration in multiple toxicity associated regions within the CH_2 domain.

5. A method for preventing immunoglobulin-induced toxicity resulting from immunotherapy for a disease in a subject comprising:

(a) selecting an immunoglobulin which recognizes and binds a target, the target being associated with the disease;

(b) mutating the immunoglobulin so selected by structurally altering multiple toxicity associated domains in the constant region of the immunoglobulin thereby creating a structurally altered immunoglobulin;

(c) administering the structurally altered immunoglobulin of step (b) to the subject under conditions so that the structurally altered immunoglobulin recognizes and binds the target thereby alleviating symptoms associated with the disease, the structural alteration of the constant region thereby preventing immunoglobulin-induced toxicity in the subject.

6. A method for preventing immunoglobulin-induced toxicity resulting from immunotherapy for a disease in a subject comprising:

(a) selecting an Ig fusion protein which recognizes and binds a target, the target being associated with the disease;

(b) structurally altering multiple toxicity associated domains in the CH_2 domain of the constant region of the Ig protein so selected;

(c) administering the structurally altered Ig fusion protein of step (b) to the subject under conditions so that the structurally altered Ig fusion protein recognizes and binds the target thereby alleviating symptoms associated with the disease, the structural alteration of the CH_2 domain thereby preventing immunoglobulin-induced toxicity in the subject.

7. The method of claim 1, 2, 3, 4, 5, or 6, wherein the portion of the constant region is the CH_2 domain.

8. The method of claim 1 or 5, wherein the immunoglobulin molecule is IgG.

9. The method of claim 1 or 5, wherein the immunoglobulin molecule is IgM.

10. The method of claim 1 or 5, wherein the immunoglobulin molecule is IgA.

11. The method of claim 2, wherein the antibody recognizes and binds Le^y.

12. The method of claim 2, wherein the antibody recognizes and binds to Le^x.

13. The method of claim 2, wherein the antibody is a monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC.

14. The method of claim 2, wherein the antibody is a chimeric antibody ChiBR96 produced by the hybridoma having the identifying characteristics of HB 10460 as deposited with the ATCC.

15. The method of claim 1 or 5, wherein the immunoglobulin recognizes and binds Le^y.

16. The method of claim 1 or 5, wherein the immunoglobulin recognizes and binds to Le^x.

17. The method of claim 1 or 5, wherein the immunoglobulin is a monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC.

18. The method of claim 1 or 5, wherein the immunoglobulin is a chimeric antibody ChiBR96 produced by the hybridoma having the identifying characteristics of HB 10460 as deposited with the ATCC.

19. The method of claim 3, 4, or 6, wherein the Ig fusion protein recognizes and binds Le^y.

20. The method of claim 3, 4, or 6, wherein the Ig fusion protein recognizes and binds to Le^x.

21. The method of claim 3, 4, or 6, wherein the Ig fusion protein is a derivative of monoclonal antibody BR96 produced by the hybridoma having the identifying characteristics of HB 10036 as deposited with the ATCC.

22. The method of claim 3, 4, or 6, wherein the Ig fusion protein is a derivative of chimeric antibody ChiBR96 produced by the hybridoma having the identifying characteristics of HB 10460 as deposited with the ATCC.

23. A pharmaceutical composition comprising a pharmaceutically effective

amount of a structurally altered immunoglobulin, and an acceptable carrier, the structurally altered immunoglobulin (1) recognizes and binds a target, the target is associated with cancer and (2) has an inactivated CH_2 domain.

24. A pharmaceutical composition comprising a pharmaceutically effective amount of structurally altered Ig fusion protein, and an acceptable carrier, the structurally altered Ig fusion protein (1) recognizes and binds a target, the target is associated with cancer and (2) has an inactivated CH_2 domain.

25. A method of treating carcinomas in vivo comprising administering to a subject a pharmaceutically effective amount of the composition of claim 23 or 24.

26. The method of claim 30, wherein the structurally altered immunoglobulin in the composition is labeled so as to directly or indirectly produce a detectable signal with a compound selected from the group consisting of a radiolabel, an enzyme, a chromophore, a chemiluminescer, and a fluorescer.

27. The method of claim 24, wherein the Ig fusion protein in the composition is labeled so as to directly or indirectly produce a detectable signal with a compound selected from the group consisting of a radiolabel, an enzyme, a chromophore, a chemiluminescer, and a fluorescer.

28. The method of claim 2 or 5, wherein the antibody is conjugated to a cytotoxic agent.

29. The method of claim 1, wherein the immunoglobulin is conjugated to a cytotoxic agent.

30. The method of claim 3, 4 or 6, wherein the Ig fusion protein is conjugated to a cytotoxic agent.

31. The method of claim 28, 29, or 31, wherein the cytotoxic agent is selected from the group consisting of antimetabolites, alkylating agents, anthracyclines, antibiotics, anti-mitotic agents, and chemotherapeutic agents.

32. A method for treating a subject suffering from a cancer, the cancer being characterized as a group of cells having a tumor associated antigen on the cell surface, which method comprises administering to the subject a cancer killing amount of the composition of claim 23 or 24 joined to a cytotoxic agent under conditions which permit the molecule so joined to bind the tumor associated antigen on the cell surface so as to kill the cells so bound thereby curing the subject.

33. A pharmaceutical composition comprising a pharmaceutically effective amount of a structurally altered BR96 antibody, the structurally altered antibody having an inactivated CH_2 domain.

34. A method for treating a subject suffering from a proliferative type disease characterized by cells having a BR96 antigen on the cell surface which comprises administering to the subject an effective amount of the composition of claim 33 joined to doxorubicin such that the immunoconjugate binds the BR96 antigen and kills said cells thereby treating the subject.

35. A method for inhibiting BR96 (ATCC: HB10036) induced toxicity resulting from immunoglobulin immunotherapy in a subject comprising administering

BR96 to the subject, the BR96 molecule being modified prior to administration, the modification comprising the deletion or substitution of at least one amino acid residue in the toxicity associated domain localized to amino acids 310-331 and the deletion or substitution of at least one amino acid residue in the toxicity associated domain localized to amino acids 231-238 so that complement and Fc receptor mediated toxicity is inhibited.

36. A method for preventing BR96 (ATCC: HB10036) induced toxicity resulting from immunotherapy for cancer in a subject comprising:

(a) mutating the BR96 polypeptide by the deletion or substitution of at least one amino acid residue in the toxicity associated domain localized to amino acids 310-331 and the deletion or substitution of at least one amino acid residue in the toxicity associated domain localized to amino acids 231-238 so that complement and Fc receptor mediated immunoglobulin-induced toxicity is inhibited in the altered BR96 polypeptide; and

(b) administering the structurally altered BR96 polypeptide of step (a) to the subject under conditions so that the peptide recognizes and binds cancer associated Le^y antigens, thereby alleviating symptoms associated with the cancer, the structural alteration of the toxicity associated domains thereby preventing BR96 toxicity in the subject.

37. A chimeric BR96 antibody having a structurally altered constant region having the CH1 and CH3 domains but not the CH2 domain, the antibody being designated cBR96-A.

38. The chimeric BR96 antibody of claim 37 which is expressed by the plasmid having the sequence shown in SEQ ID NO. 10.

39. A BR96 antibody having humanized variable and constant regions, wherein the constant region has been structurally altered so that the CH1 and CH3 domains are present but the CH2 domain is not, the antibody being designated hBR96-2A.

40. The BR96 antibody of claim 39 which is expressed by the plasmid having the sequence shown in SEQ ID NO. 12.

41. A BR96 antibody designated hBR96-2B having a structurally altered constant region wherein leucine at amino acid position 235 is mutated to alanine and glycine at amino acid position 237 is mutated to alanine.

42. A BR96 antibody designated hBR96-2C having a structurally altered constant region wherein glutamic acid at amino acid position 318 is mutated to serine; lysine at amino acid position 320 is mutated to serine; and lysine at amino acid position 322 is mutated to serine.

43. A BR96 antibody designated hBR96-2D having a structurally altered constant region wherein proline at amino acid position 331 is mutated to alanine.

44. A BR96 antibody designated hBR96-2E having a structurally altered constant region wherein leucine at amino acid position 235 is mutated to alanine; glycine at amino acid position 237 is mutated to alanine; glutamic acid at amino acid position 318 is mutated to serine; lysine at amino acid

position 320 is mutated to serine; and lysine at amino acid position 322 is mutated to serine.

45. A BR96 antibody designated hBR96-2F having a structurally altered constant region wherein leucine at amino acid position 235 is mutated to alanine; glycine at amino acid position 237 is mutated to alanine; and proline at amino acid position 331 is mutated to alanine.

46. A BR96 antibody designated hBR96-2G having a structurally altered constant region wherein glutamic acid at amino acid position 318 is mutated to serine; lysine at amino acid position 320 is mutated to serine; and lysine at amino acid position 322 is mutated to serine; and proline at amino acid position 331 is mutated to alanine.

47. A BR96 antibody designated hBR96-2H having a structurally altered constant region wherein leucine at amino acid position 235 is mutated to alanine; glycine at amino acid position 237 is mutated to alanine; glutamic acid at amino acid position 318 is mutated to serine; lysine at amino acid position 320 is mutated to serine; lysine at amino acid position 322 is mutated to serine; and proline at amino acid position 331 is mutated to alanine.

48. A nucleic acid molecule which encodes the BR96 antibody of claim 37, 39, and 41-47.

49. A cDNA of claim 48.

50. A plasmid which comprises the nucleic acid molecule of claim 48.

51. A host vector system comprising a plasmid of claim 50 in a suitable host cell.

52. A method for producing a protein comprising growing the host vector system of claim 51 so as to produce the protein in the host and recovering the protein so produced.

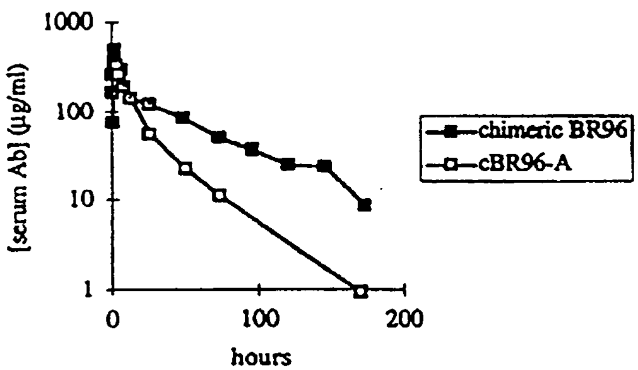


Figure 1. Plasma clearance in high LeY expressing dogs chimeric versus constant region mutant of cBR96-2.

Figure One

Figure 2

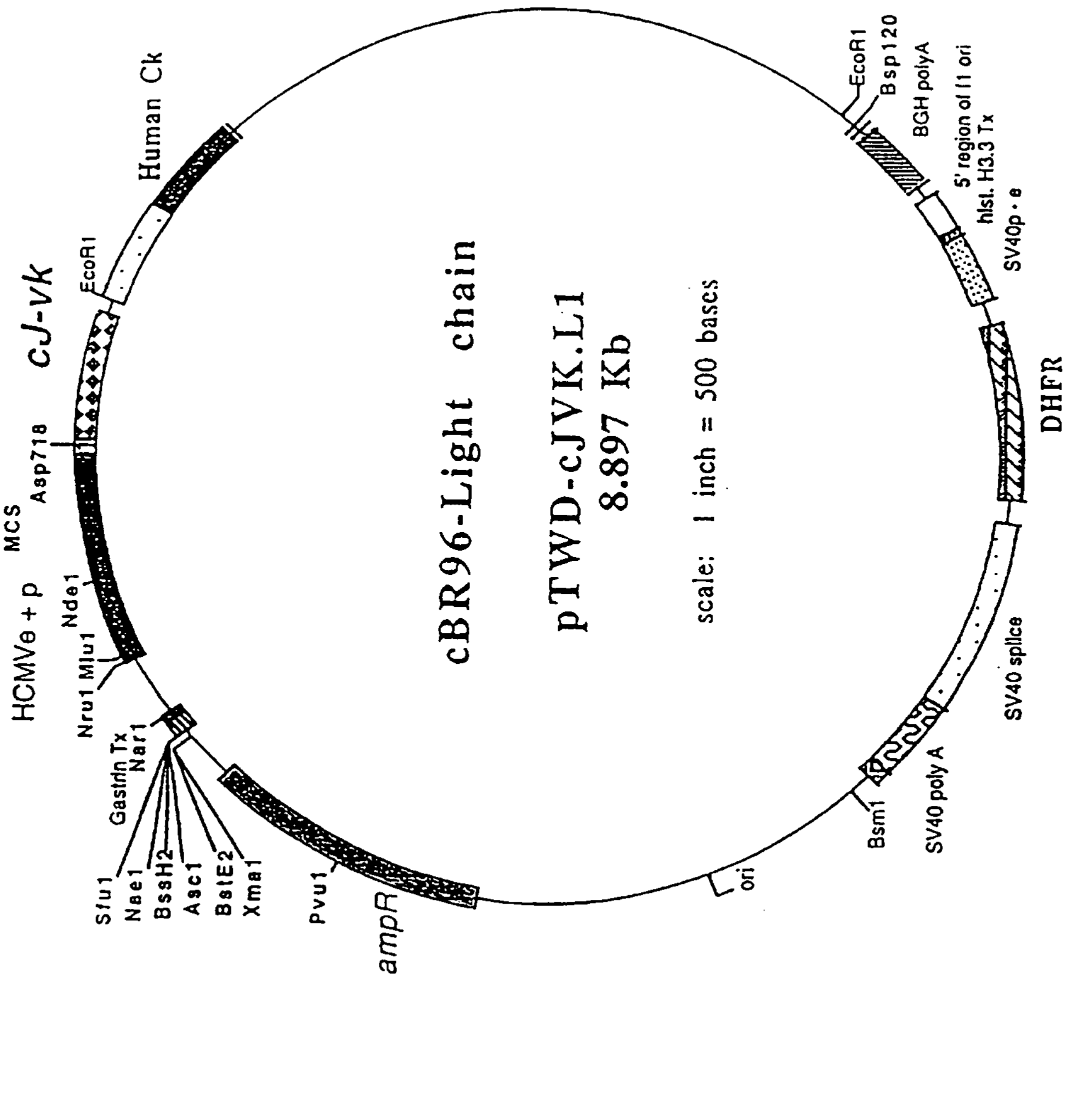

cBR96-Light chain
pTWD-cJVK.L1
8.897 Kb
scale: 1 inch = 500 bases
Human Ck
EcoR1
cJ-vk
Asp718
MCS
HCMVe + p
Nde1
Nru1 Mlu1
Gastrin Tx
Nar1
Sfu1
Nae1
BssH2
Asc1
BstE2
Xma1
Pvu1
ampR
ori
Bsm1
SV40 poly A
SV40 splice
DHFR
SV40p - e
5' region of f1 ori
hist. H3.3 Tx
BGH polyA
Bsp120
EcoR1

Figure 3

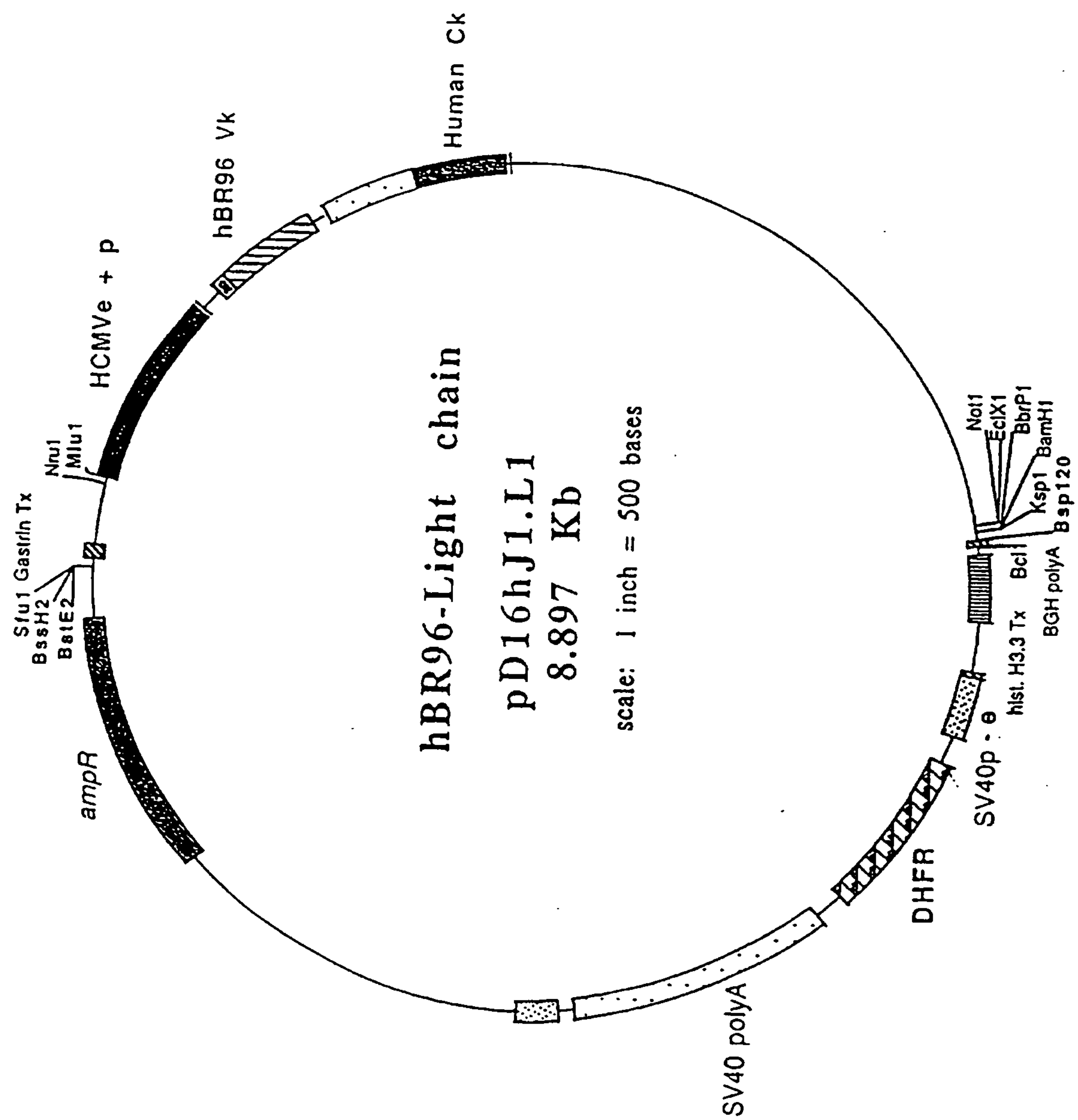

hBR96-Light chain
pD16hJ1.L1
8.897 Kb
scale: 1 inch = 500 bases
Human Ck
hBR96 Vk
HCMVe + p
Nru1
Mlu1
Sfu1
BssH2
BstE2
Gastrin Tx
ampR
SV40 polyA
DHFR
SV40p - e
hist. H3.3 Tx
BGH polyA
Bcl1
Not1
EclX1
BbrP1
BamH1
Ksp1
Bsp120

Figure 4

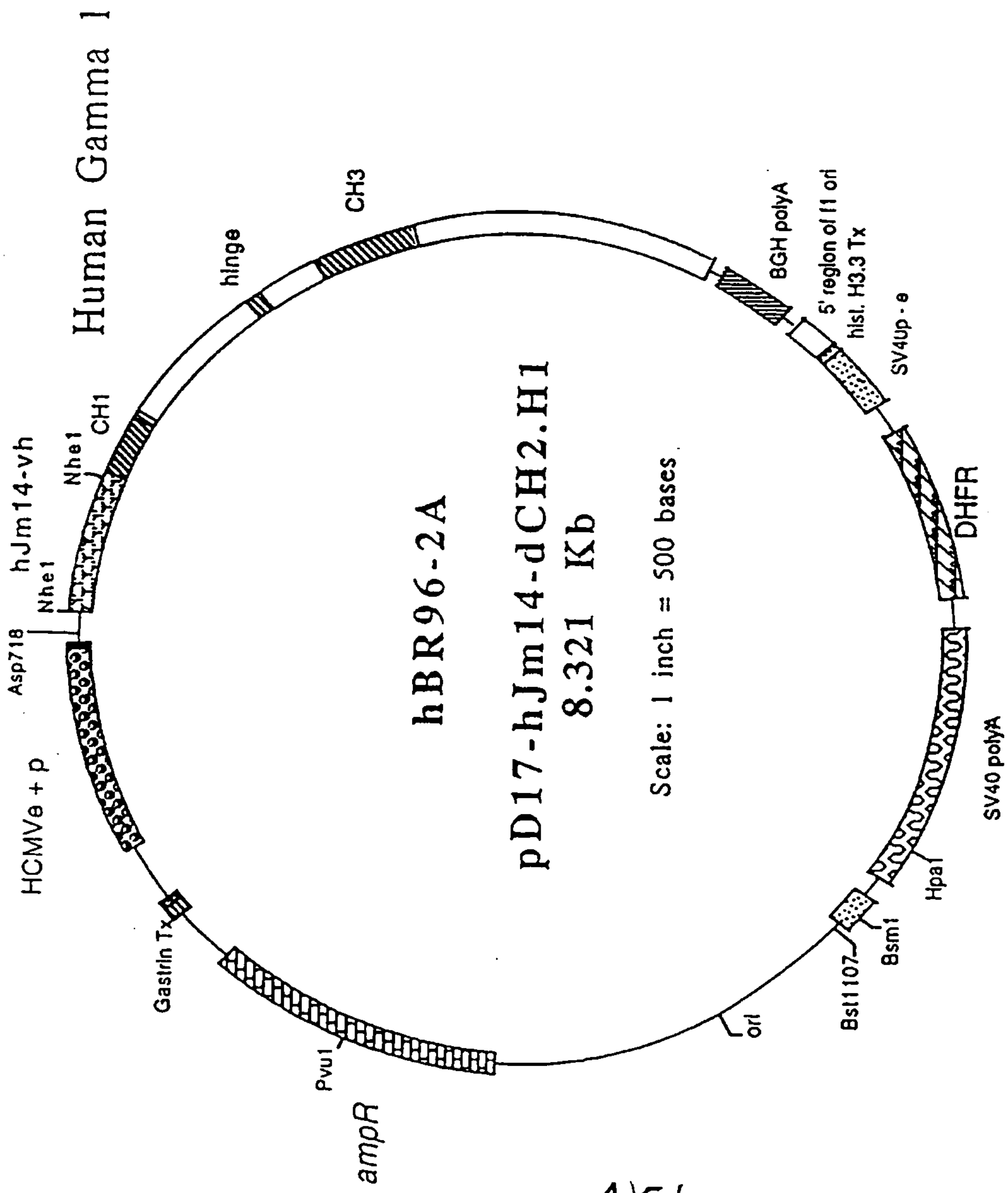
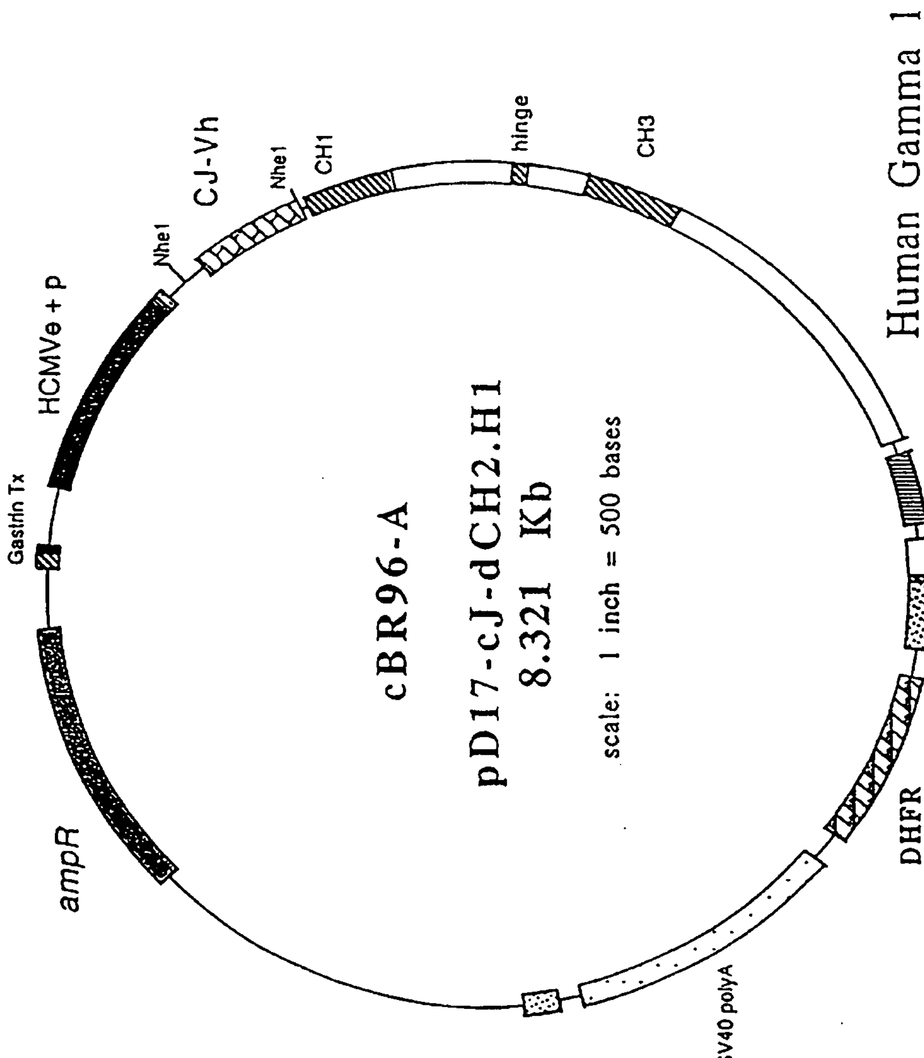

Human Gamma 1
hBR96-2A
pD17-hJm14-dCH2.H1
8.321 Kb
Scale: 1 inch = 500 bases
CH3
hinge
CH1
Nhe1
hJm14-vh
Nhe1
Asp718
HCMVe + p
Gastrin Tx
Pvu1
ampR
ori
Bst1107
Bsm1
Hpa1
SV40 polyA
DHFR
SV4Up - e
hist. H3.3 Tx
5' region of f1 ori
BGH polyA

Figure 5



cBR96-A
pD17-cJ-dCH2.H1
8.321 Kb
scale: 1 inch = 500 bases
Human Gamma 1
CJ-Vh
Nhe1
Nhe1
CH1
hinge
CH3
HCMVe + p
Gastrin Tx
ampR
SV40 polyA
DHFR
SV40p - e
BGH polyA
hlsl. H3.3 Tx

Figure 6

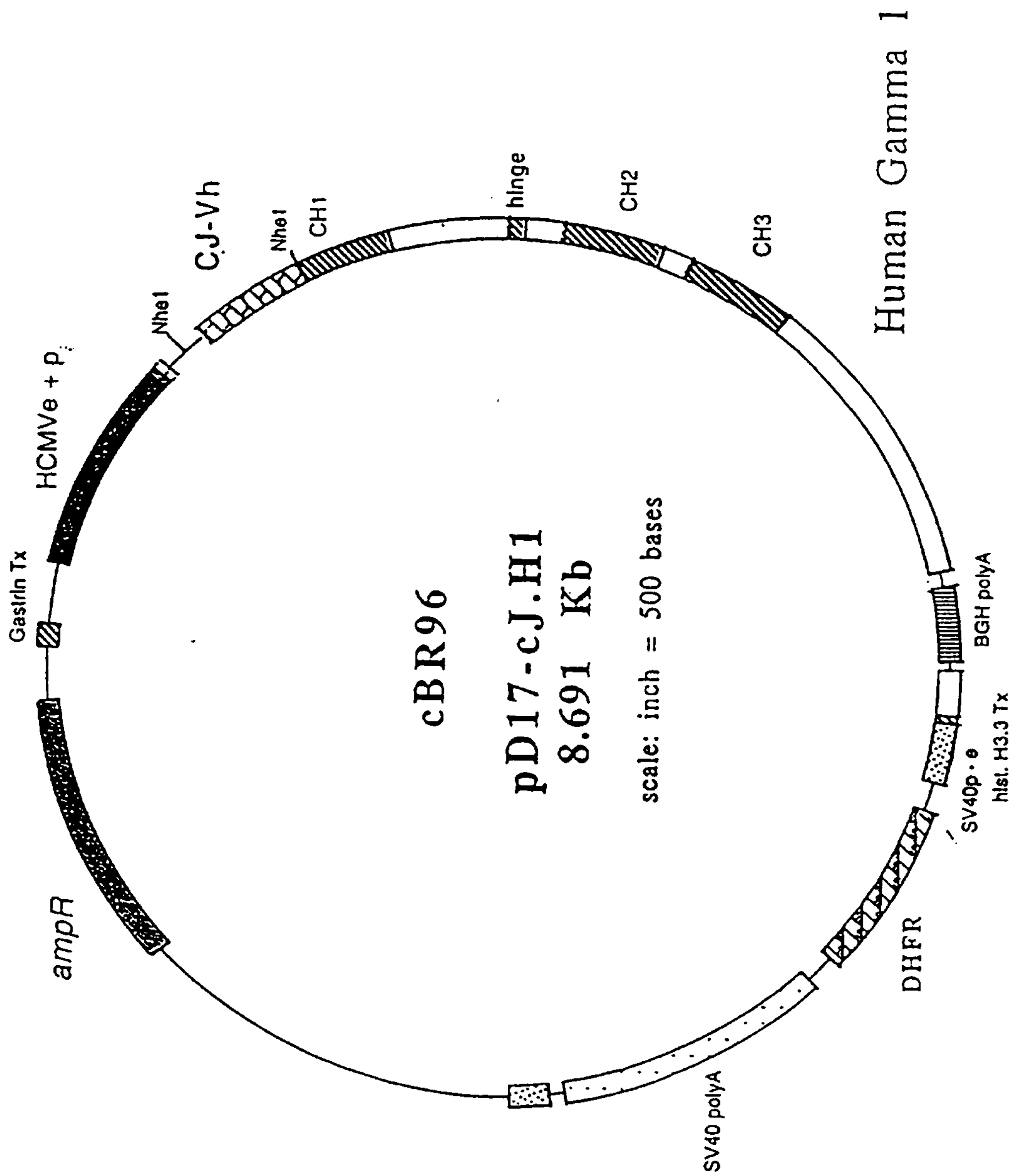

cBR96
pD17-cJ.H1
8.691 Kb
scale: inch = 500 bases
Human Gamma 1
CJ-Vh
Nhe1
CH1
hinge
CH2
CH3
BGH polyA
hist. H3.3 Tx
SV40p - e
DHFR
SV40 polyA
ampR
Gastrin Tx
HCMVe + p

Figure 7

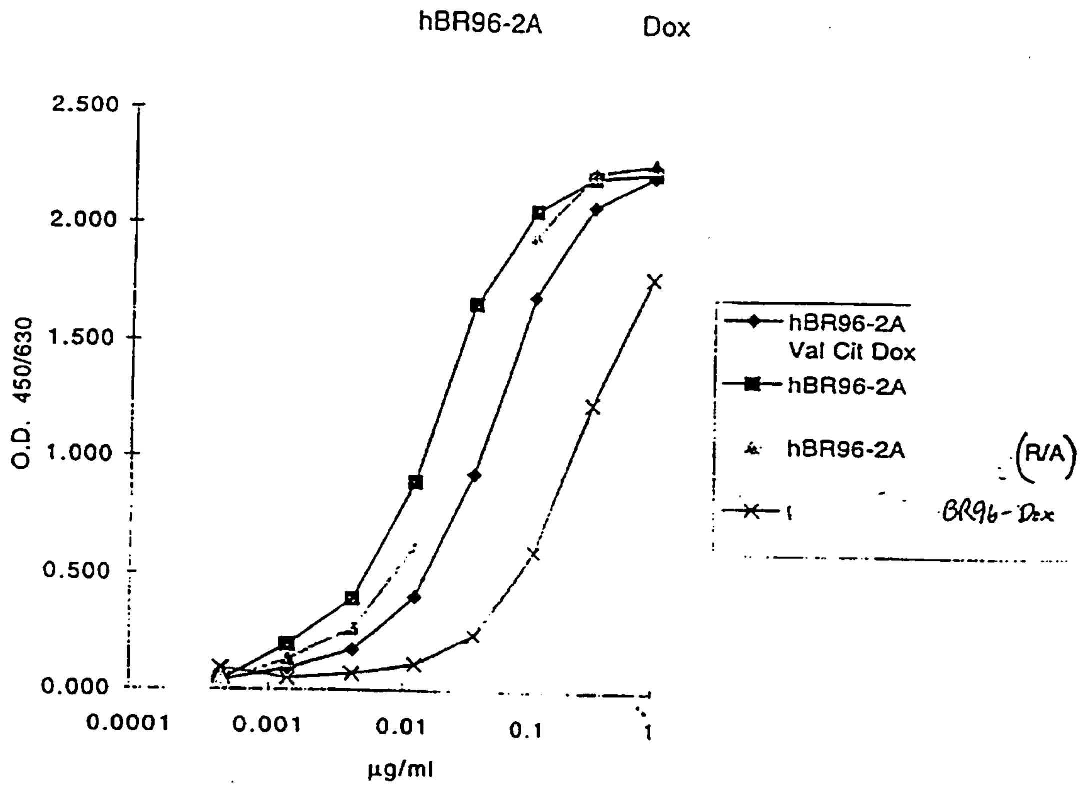
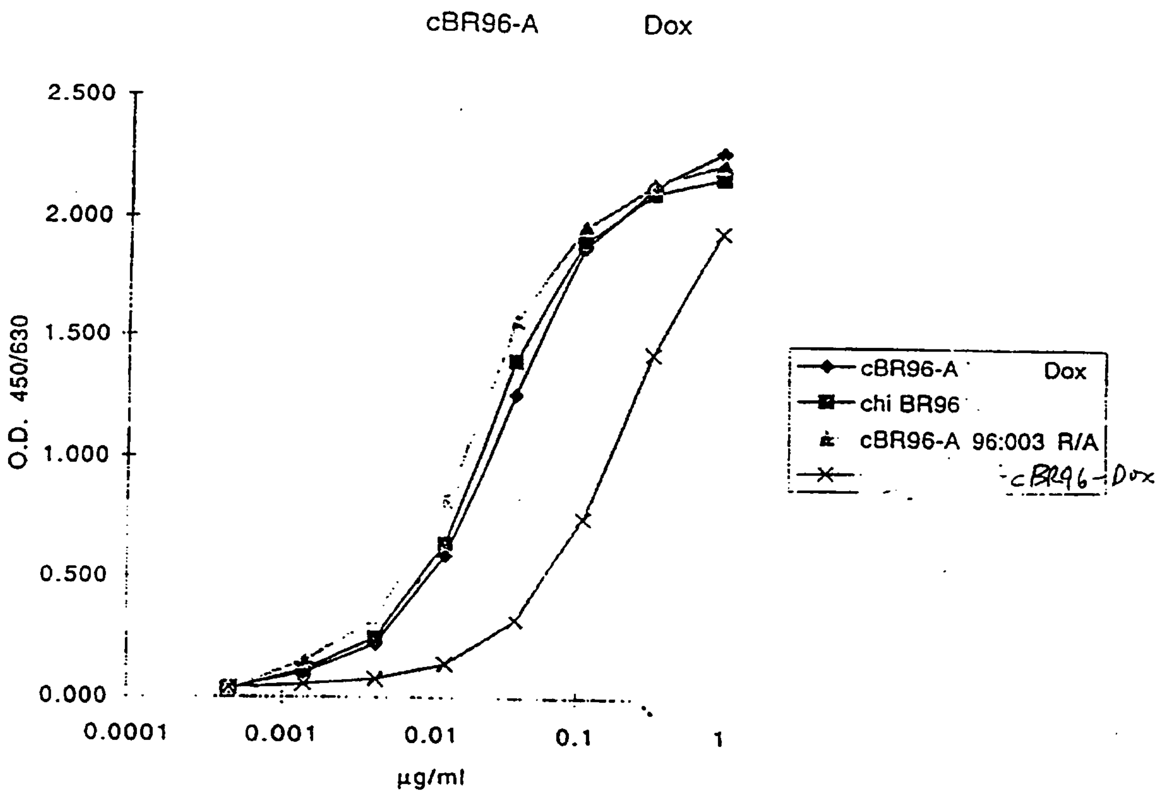
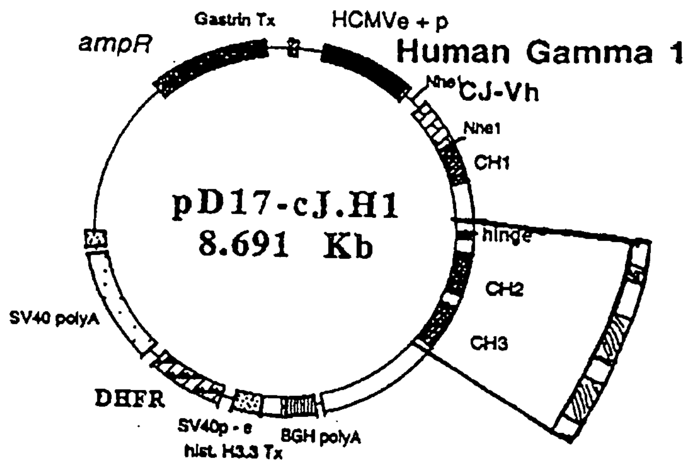

hBR96-2A Dox
2.500
2.000
1.500
1.000
0.500
0.000
O.D. 450/630
0.0001
0.001
0.01
0.1
1
µg/ml
hBR96-2A
Val Cit Dox
hBR96-2A
hBR96-2A
(R/A)
BR96-Dox

Figure 8


cBR96-A Dox
2.500
2.000
1.500
1.000
0.500
0.000
O.D. 450/630
0.0001
0.001
0.01
0.1
1
µg/ml
cBR96-A Dox
chi BR96
cBR96-A 96:003 R/A
cBR96-Dox

A- Hinge + CL + H3 domains were removed from R96 IgG1 construct by E.co -III restriction digestion.



B. 2 - Hinge + CH3 domains amplified by PCR from L6 IgG1 construct lacking the CH2 domain.

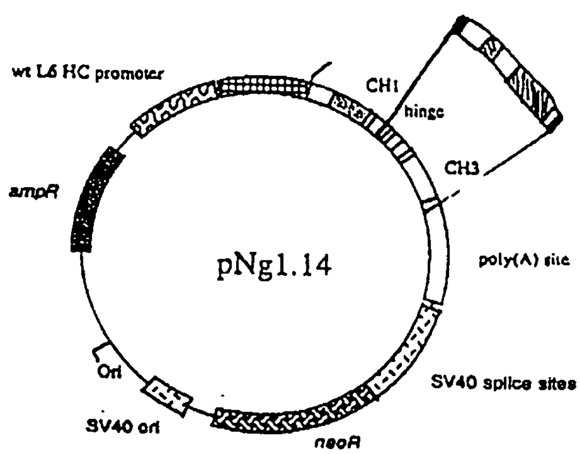


Figure 9

9B - Hinge +CH3 PCR fragment cloned by homologous recombination into E.co47-III site of BR96 IgG1 molecule.

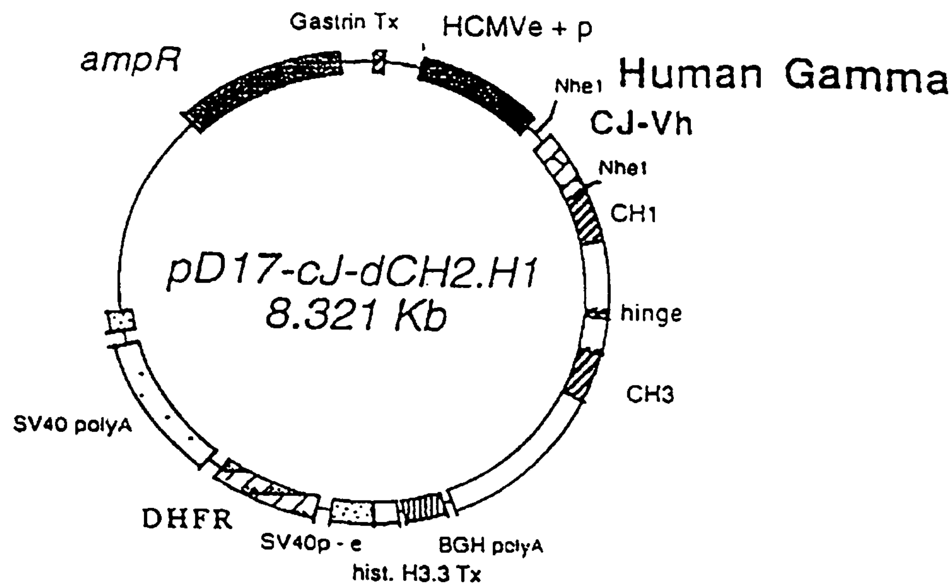
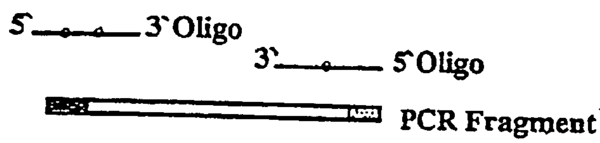


Figure 9

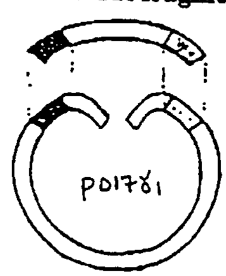
(CONTINUED)

1- Introduction of mutations by site-directed mutagenesis on double-stranded plasmid DNA.

A- Mutations introduced into synthetic oligonucleotides used for the PCR amplification of CH2 domain.



B- Plasmid DNA linearized inside CH2 domain and co-transformed with PCR fragment into competent DH5α.



C- Cloning mediated by homologous recombination yields transformants harbouring recombinant plasmids.

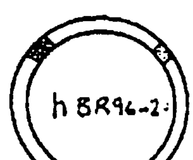
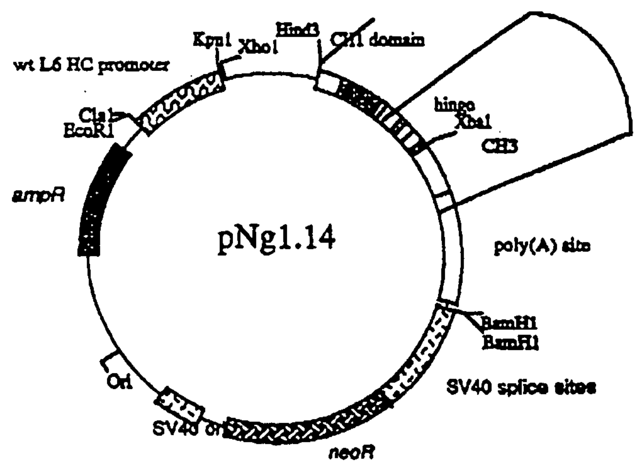


Figure 10

Figure 11


pNg1.14
wt L6 HC promoter
Kpn1
Xho1
Hind3
CH1 domain
hinge
Xba1
CH3
Cla1
EcoR1
ampR
poly(A) site
BamH1
BamH1
SV40 splice sites
Ori
SV40 ori
neoR

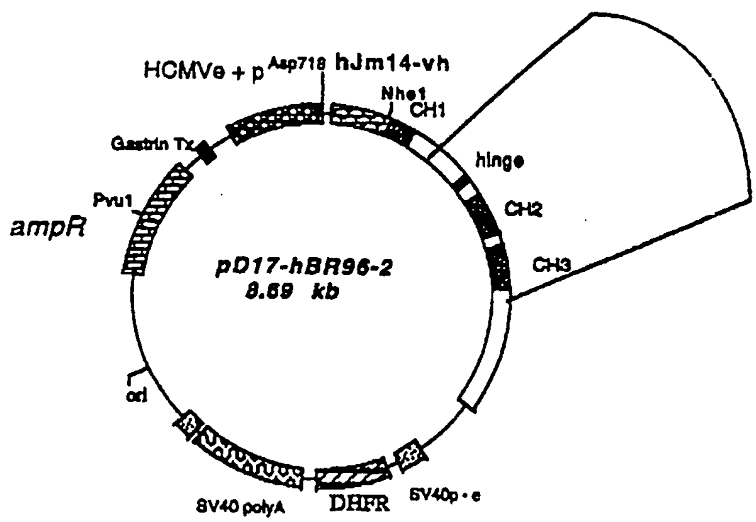

HCMVe + p
Asp718
hJm14-vh
Nhe1
CH1
hinge
CH2
CH3
Gastrin Tx
Pvu1
ampR
pD17-hBR96-2
8.69 kb
ori
SV40 polyA
DHFR
SV40p • e

Figure 12

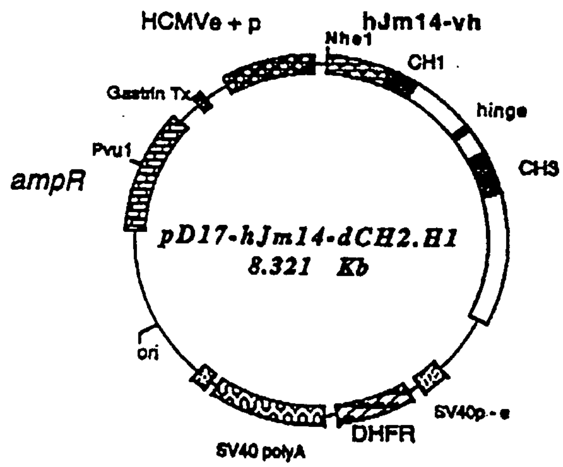

HCMVe + p
hJm14-vh
Nhe1
CH1
hinge
CH3
Gastrin Tx
Pvu1
ampR
pD17-hJm14-dCH2.H1
8.321 Kb
ori
SV40p - e
DHFR
SV40 polyA

Figure 13

pD17-cJ-dCH2.H1

```
        10         20         30         40         50         60         70         80         90
GACGGATCGG GAGATCTGCT AGGTGACCTG AGGCGCGCCG GCTTCGAATA GCCAGAGTAA CCTTTTTTTT TAATTTTATT TTATTTTATT
CTGCCTAGCC CTCTAGACGA TCCACTGGAC TCCGCGCGGC CGAAGCTTAT CGGTCTCATT GGAAAAAAAA ATTAAAATAA AATAAAATAA

       100        110        120        130        140        150        160        170        180
TTTGAGATGG AGTTTGGCGC CGATCTCCCG ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAGTATC
AAACTCTACC TCAAACCGCG GCTAGAGGGC TAGGGGATAC CAGCTGAGAG TCATGTTAGA CGAGACTACG GCGTATCAAT TCGGTCATAG

       190        200        210        220        230        240        250        260        270
TGCTCCCTGC TTGTGTGTTG GAGGTCGCTG AGTAGTGCGC GAGCAAAATT TAAGCTACAA CAAGGCAAGG CTTGACCGAC AATTGCATGA
ACGAGGGACG AACACACAAC CTCCAGCGAC TCATCACGCG CTCGTTTTAA ATTCGATGTT GTTCCGTTCC GAACTGGCTG TTAACGTACT

       280        290        300        310        320        330        340        350        360
AGAATCTGCT TAGGGTTAGG CGTTTTGCGC TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT AGTTATTAAT
TCTTAGACGA ATCCCAATCC GCAAAACGCG ACGAAGCGCT ACATGCCCGG TCTATATGCG CAACTGTAAC TAATAACTGA TCAATAATTA

       370        380        390        400        410        420        430        440        450
AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC TTACGGTAAA TGGCCCGCCT GGCTGACCGC
TCATTAGTTA ATGCCCCAGT AATCAAGTAT CGGGTATATA CCTCAAGGCG CAATGTATTG AATGCCATTT ACCGGGCGGA CCGACTGGCG

       460        470        480        490        500        510        520        530        540
CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA TGGGTGGACT
GGTTGCTGGG GGCGGGTAAC TGCAGTTATT ACTGCATACA AGGGTATCAT TGCGGTTATC CCTGAAAGGT AACTGCAGTT ACCCACCTGA

       550        560        570        580        590        600        610        620        630
ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG
TAAATGCCAT TTGACGGGTG AACCGTCATG TAGTTCACAT AGTATACGGT TCATGCGGGG GATAACTGCA GTTACTGCCA TTTACCGGGC

       640        650        660        670        680        690        700        710        720
CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC
GGACCGTAAT ACGGGTCATG TACTGGAATA CCCTGAAAGG ATGAACCGTC ATGTAGATGC ATAATCAGTA GCGATAATGG TACCACTACG

       730        740        750        760        770        780        790        800        810
GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT
CCAAAACCGT CATGTAGTTA CCCGCACCTA TCGCCAAACT GAGTGCCCCT AAAGGTTCAG AGGTGGGGTA ACTGCAGTTA CCCTCAAACA

       820        830        840        850        860        870        880        890        900
TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG
AAACCGTGGT TTTAGTTGCC CTGAAAGGTT TTACAGCATT GTTGAGGCGG GGTAACTGCG TTTACCCGCC ATCCGCACAT GCCACCCTCC
```

Figure 14

15156

pD17-cJ-dCH2.H1

```
       910        920        930        940        950        960        970        980        990
TCTATATAAG CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTACTGG CTTATCGAAA TTAATACGAC TCACTATAGG GAGACCCAAG
AGATATATTC GTCTCGAGAG ACCGATTGAT CTCTTGGGTG ACGAATGACC GAATAGCTTT AATTATGCTG AGTGATATCC CTCTGGGTTC

      1000       1010       1020       1030       1040       1050       1060       1070       1080
CTTGGTACCA ATTTAAATTG ATATCTCCTT AGGTCTCGAG TCTCTAGATA ACCGGTCAAT CGATTGGAAT TCTTGCGGCC GCTTGCTAGC
GAACCATGGT TAAATTTAAC TATAGAGGAA TCCAGAGCTC AGAGATCTAT TGGCCAGTTA GCTAACCTTA AGAACGCCGG CGAACGATCG

      1090       1100       1110       1120       1130       1140       1150       1160       1170
CACCATGGAG TTGTGGTTAA GCTTGGTCCT TCCTTGTCCT TGTTTTAAAA GGTGTCCAGT GTGAAGTGAA TCTGGTGGAG TCTGGGGGAG
GTGGTACCTC AACACCAATT CGAACCAGGA AGGAACAGGA ACAAAATTTT CCACAGGTCA CACTTCACTT AGACCACCTC AGACCCCCTC

      1180       1190       1200       1210       1220       1230       1240       1250       1260
GCTTAGTGCA GCCTGGAGGG TCCCTGAAAG TCTCCTGTGT AACCTCTGGA TTCACTTTCA GTGACTATTA CATGTATTGG GTTCGCCAGA
CGAATCACGT CGGACCTCCC AGGGACTTTC AGAGGACACA TTGGAGACCT AAGTGAAAGT CACTGATAAT GTACATAACC CAAGCGGTCT

      1270       1280       1290       1300       1310       1320       1330       1340       1350
CTCCAGAGAA GAGGCTGGAG TGGGTCGCAT ACATTAGTCA AGGTGGTGAT ATAACCGACT ATCCAGACAC TGTAAAGGGT CGATTCACCA
GAGGTCTCTT CTCCGACCTC ACCCAGCGTA TGTAATCAGT TCCACCACTA TATTGGCTGA TAGGTCTGTG ACATTTCCCA GCTAAGTGGT

      1360       1370       1380       1390       1400       1410       1420       1430       1440
TCTCCAGAGA CAATGCCAAG AACACCCTGT ACCTGCAAAT GAGCCGTCTG AAGTCTGAGG ACACAGCCAT GTATTACTGT GCAAGAGGCC
AGAGGTCTCT GTTACGGTTC TTGTGGGACA TGGACGTTTA CTCGGCAGAC TTCAGACTCC TGTGTCGGTA CATAATGACA CGTTCTCCGG

      1450       1460       1470       1480       1490       1500       1510       1520       1530
TGGACGACGG GGCCTGGTTT GCTTACTGGG GCCAAGGGAC TCTGGTCACG GTCTCTGTAG CTAGCACCAA GGGCCCATCG GTCTTCCCCC
ACCTGCTGCC CCGGACCAAA CGAATGACCC CGGTTCCCTG AGACCAGTGC CAGAGACATC GATCGTGGTT CCCGGGTAGC CAGAAGGGGG

      1540       1550       1560       1570       1580       1590       1600       1610       1620
TGGCACCCTC CTCCAAGAGC ACCTCTGGGG GCACAGCGGC CCTGGGCTGC CTGGTCAAGG ACTACTTCCC CGAACCGGTG ACGGTGTCGT
ACCGTGGGAG GAGGTTCTCG TGGAGACCCC CGTGTCGCCG GGACCCGACG GACCAGTTCC TGATGAAGGG GCTTGGCCAC TGCCACAGCA

      1630       1640       1650       1660       1670       1680       1690       1700       1710
GGAACTCAGG CGCCCTGACC AGCGGCGTGC ACACCTTCCC GGCTGTCCTA CAGTCCTCAG GACTCTACTC CCTCAGCAGC GTGGTCACCG
CCTTGAGTCC GCGGGACTGG TCGCCGCACG TGTGGAAGGG CCGACAGGAT GTCAGGAGTC CTGAGATGAG GGAGTCGTCG CACCAGTGGC

      1720       1730       1740       1750       1760       1770       1780       1790       1800
TGCCCTCCAG CAGCTTGGGC ACCCAGACCT ACATCTGCAA CGTGAATCAC AAGCCCAGCA ACACCAAGGT GGACAAGAAA GTTGGTGAGA
ACGGGAGGTC GTCGAACCCG TGGGTCTGGA TGTAGACGTT GCACTTAGTG TTCGGGTCGT TGTGGTTCCA CCTGTTCTTT CAACCACTCT
```

Figure 14
(continued)

pD17-cJ-dCH2.H1

```
      1810       1820       1830       1840       1850       1860       1870       1880       1890
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CCGGTCGTGT CCCTCCCTCC CACAGACGAC CTTCGGTCCG AGTCGCGAGG ACGGACCTGC GTAGGGCCGA TACGTCGGGG TCAGGTCCCG

      1900       1910       1920       1930       1940       1950       1960       1970       1980
AGCAAGGCAG GCCCCGTCTG CCTCTTCACC CGGAGGCCTC TGCCCGCCCC ACTCATGCTC AGGGAGAGGG TCTTCTGGCT TTTTCCCCAG
TCGTTCCGTC CGGGGCAGAC GGAGAAGTGG GCCTCCGGAG ACGGGCGGGG TGAGTACGAG TCCCTCTCCC AGAAGACCGA AAAAGGGGTC

      1990       2000       2010       2020       2030       2040       2050       2060       2070
GCTCTGGGCA GGCACAGGCT AGGTGCCCCT AACCCAGGCC CTGCACACAA AGGGGCAGGT GCTGGGCTCA GACCTGCCAA GAGCCATATC
CGAGACCCGT CCGTGTCCGA TCCACGGGGA TTGGGTCCGG GACGTGTGTT TCCCCGTCCA CGACCCGAGT CTGGACGGTT CTCGGTATAG

      2080       2090       2100       2110       2120       2130       2140       2150       2160
CGGGAGGACC CTGCCCCTGA CCTAAGCCCA CCCCAAAGGC CAAACTCTCC ACTCCCTCAG CTCGGACACC TTCTCTCCTC CCAGATTCCA
GCCCTCCTGG GACGGGGACT GGATTCGGGT GGGGTTTCCG GTTTGAGAGG TGAGGGAGTC GAGCCTGTGG AAGAGAGGAG GGTCTAAGGT

      2170       2180       2190       2200       2210       2220       2230       2240       2250
GTAACTCCCA ATCTTCTCTC TGCAGAGCCC AAATCTTGTG ACAAAACTCA CACATGCCCA CCGTGCCCAG GTAAGCCAGC CCAGGCCTCG
CATTGAGGGT TAGAAGAGAG ACGTCTCGGG TTTAGAACAC TGTTTTGAGT GTGTACGGGT GGCACGGGTC CATTCGGTCG GGTCCGGAGC

      2260       2270       2280       2290       2300       2310       2320       2330       2340
CCCTCCAGCT CAAGGCGGGA CAGGTGCCCT AGAGTAGCCT GCATCCAGGG ACACACCACG TGGGTACCAA CATGTCCGGA GCCACATGGA
GGGAGGTCGA GTTCCGCCCT GTCCACGGGA TCTCATCGGA CGTAGGTCCC TGTGTGGTGC ACCCATGGTT GTACAGGCCT CGGTGTACCT

      2350       2360       2370       2380       2390       2400       2410       2420       2430
CAGAGGCCGG CTCGGCCCAC CCTCTGCCCT GAGAGTGACC GCTGTACCAA CCTCTGTCCC TACAGGGCAG CCCCGAGAAC CACAGGTGTA
GTCTCCGGCC GAGCCGGGTG GGAGACGGGA CTCTCACTGG CGACATGGTT GGAGACAGGG ATGTCCCGTC GGGGCTCTTG GTGTCCACAT

      2440       2450       2460       2470       2480       2490       2500       2510       2520
CACCCTGCCC CCATCCCGGG ATGAGCTGAC CAAGAACCAG GTCAGCCTGA CCTGCCTGGT CAAAGGCTTC TATCCCAGCG ACATCGCCGT
GTGGGACGGG GGTAGGGCCC TACTCGACTG GTTCTTGGTC CAGTCGGACT GGACGGACCA GTTTCCGAAG ATAGGGTCGC TGTAGCGGCA

      2530       2540       2550       2560       2570       2580       2590       2600       2610
GGAGTGGGAG AGCAATGGGC AGCCGGAGAA CAACTACAAG ACCACGCCTC CCGTGCTGGA CTCCGACGGC TCCTTCTTCC TCTACAGCAA
CCTCACCCTC TCGTTACCCG TCGGCCTCTT GTTGATGTTC TGGTGCGGAG GGCACGACCT GAGGCTGCCG AGGAAGAAGG AGATGTCGTT

      2620       2630       2640       2650       2660       2670       2680       2690       2700
GCTCACCGTG GACAAGAGCA GGTGGCAGCA GGGGAACGTC TTCTCATGCT CCGTGATGCA TGAGGCTCTG CACAACCACT ACACGCAGAA
CGAGTGGCAC CTGTTCTCGT CCACCGTCGT CCCCTTGCAG AAGAGTACGA GGCACTACGT ACTCCGAGAC GTGTTGGTGA TGTGCGTCTT
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Figure 14
(continued)

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      2710       2720       2730       2740       2750       2760       2770       2780       2790
GAGCCTCTCC CTGTCTCCGG GTAAATGAGT GCGACGGCCG GCAAGCCCCC GCTCCCCGGG CTCTCGCGGT CGCACGAGGA TGCTTGGCAC
CTCGGAGAGG GACAGAGGCC CATTTACTCA CGCTGCCGGC CGTTCGGGGG CGAGGGGCCC GAGAGCGCCA GCGTGCTCCT ACGAACCGTG

      2800       2810       2820       2830       2840       2850       2860       2870       2880
GTACCCCCTG TACATACTTC CCGGGCGCCC AGCATGGAAA TAAAGCACCC AGCGCTGCCC TGGGCCCCTG CGAGACTGTG ATGGTTCTTT
CATGGGGGAC ATGTATGAAG GGCCCGCGGG TCGTACCTTT ATTTCGTGGG TCGCGACGGG ACCCGGGGAC GCTCTGACAC TACCAAGAAA

      2890       2900       2910       2920       2930       2940       2950       2960       2970
CCACGGGTCA GGCCGAGTCT GAGGCCTGAG TGGCATGAGG GAGGCAGAGC GGGTCCCACT GTCCCCACAC TGGCCCAGGC TGTGCAGGTG
GGTGCCCAGT CCGGCTCAGA CTCCGGACTC ACCGTACTCC CTCCGTCTCG CCCAGGGTGA CAGGGGTGTG ACCGGGTCCG ACACGTCCAC

      2980       2990       3000       3010       3020       3030       3040       3050       3060
TGCCTGGGCC CCCTAGGGTG GGGCTCAGCC AGGGGCTGCC CTCGGCAGGG TGGGGGATTT GCCAGCGTGG CCCTCCCTCC AGCAGCACCT
ACGGACCCGG GGGATCCCAC CCCGAGTCGG TCCCCGACGG GAGCCGTCCC ACCCCCTAAA CGGTCGCACC GGGAGGGAGG TCGTCGTGGA

      3070       3080       3090       3100       3110       3120       3130       3140       3150
GCCCTGGGCT GGGCCACGGG AAGCCCTAGG AGCCCCTGGG GACAGACACA CAGCCCCTGC CTCTGTAGGA GACTGTCCTG TTCTGTGAGC
CGGGACCCGA CCCGGTGCCC TTCGGGATCC TCGGGGACCC CTGTCTGTGT GTCGGGGACG GAGACATCCT CTGACAGGAC AAGACACTCG

      3160       3170       3180       3190       3200       3210       3220       3230       3240
GCCCCTGTCC TCCCGACCTC CATGCCCACT CGGGGGCATG CCTAGTCCAT GTGCGTAGGG ACAGGCCCTC CCTCACCCAT CTACCCCCAC
CGGGGACAGG AGGGCTGGAG GTACGGGTGA GCCCCCGTAC GGATCAGGTA CACGCATCCC TGTCCGGGAG GGAGTGGGTA GATGGGGGTG

      3250       3260       3270       3280       3290       3300       3310       3320       3330
GGCACTAACC CCTGGCTGCC CTGCCCAGCC TCGCACCCGC ATGGGGACAC AACCGACTCC GGGGACATGC ACTCTCGGGC CCTGTGGAGG
CCGTGATTGG GGACCGACGG GACGGGTCGG AGCGTGGGCG TACCCCTGTG TTGGCTGAGG CCCCTGTACG TGAGAGCCCG GGACACCTCC

      3340       3350       3360       3370       3380       3390       3400       3410       3420
GACTGGTGCA GATGCCCACA CACACACTCA GCCCAGACCC GTTCAACAAA CCCCGCACTG AGGTTGGCCG GCCACACGGC CACCACACAC
CTGACCACGT CTACGGGTGT GTGTGTGAGT CGGGTCTGGG CAAGTTGTTT GGGGCGTGAC TCCAACCGGC CGGTGTGCCG GTGGTGTGTG

      3430       3440       3450       3460       3470       3480       3490       3500       3510
ACACGTGCAC GCCTCACACA CGGAGCCTCA CCCGGGCGAA CTGCACAGCA CCCAGACCAG AGCAAGGTCC TCGCACACGT GAACACTCCT
TGTGCACGTG CGGAGTGTGT GCCTCGGAGT GGGCCCGCTT GACGTGTCGT GGGTCTGGTC TCGTTCCAGG AGCGTGTGCA CTTGTGAGGA

      3520       3530       3540       3550       3560       3570       3580       3590       3600
CGGACACAGG CCCCCACGAG CCCCACGCGG CACCTCAAGG CCCACGAGCC TCTCGGCAGC TTCTCCACAT GCTGACCTGC TCAGACAAAC
GCCTGTGTCC GGGGGTGCTC GGGGTGCGCC GTGGAGTTCC GGGTGCTCGG AGAGCCGTCG AAGAGGTGTA CGACTGGACG AGTCTGTTTG
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Figure 14
(continued)

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      3610       3620       3630       3640       3650       3660       3670       3680       3690
CCAGCCCTCC TCTCACAAGG GTGCCCCTGC AGCCGCCACA CACACACAGG GGATCACACA CCACGTCACG TCCCTGGCCC TGGCCCACTT
GGTCGGGAGG AGAGTGTTCC CACGGGGACG TCGGCGGTGT GTGTGTGTCC CCTAGTGTGT GGTGCAGTGC AGGGACCGGG ACCGGGTGAA

      3700       3710       3720       3730       3740       3750       3760       3770       3780
CCCAGTGCCG CCCTTCCCTG CAGGACGGAT CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT TTGCCCCTCC CCCGTGCCTT
GGGTCACGGC GGGAAGGGAC GTCCTGCCTA GTCGGAGCTG ACACGGAAGA TCAACGGTCG GTAGACAACA AACGGGGAGG GGGCACGGAA

      3790       3800       3810       3820       3830       3840       3850       3860       3870
CCTTGACCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC
GGAACTGGGA CCTTCCACGG TGAGGGTGAC AGGAAAGGAT TATTTTACTC CTTTAACGTA GCGTAACAGA CTCATCCACA GTAAGATAAG

      3880       3890       3900       3910       3920       3930       3940       3950       3960
TGGGGGGTGG GGTGGGGCAG GACAGCAAGG GGGAGGATTG GGAAGACAAT AGCAGGCATG CTGGGGATGC GGTGGGCTCT ATGGCTTCTG
ACCCCCCACC CCACCCCGTC CTGTCGTTCC CCCTCCTAAC CCTTCTGTTA TCGTCCGTAC GACCCCTACG CCACCCGAGA TACCGAAGAC

      3970       3980       3990       4000       4010       4020       4030       4040       4050
AGGCGGAAAG AACCAGCTGG GGCTCTAGGG GGTATCCCCA CGCGCCCTGT AGCGGCGCAT TAAGCGCGGC GGGTGTGGTG GTTACGCGCA
TCCGCCTTTC TTGGTCGACC CCGAGATCCC CCATAGGGGT GCGCGGGACA TCGCCGCGTA ATTCGCGCCG CCCACACCAC CAATGCGCGT

      4060       4070       4080       4090       4100       4110       4120       4130       4140
GCGTGACCGC TACACTTGCC AGCGCCCTAG CGCCCGCTCC TTTCGCTTTC TTCCCTTCCT TTCTCGCCAC GTTCGCCGGG CCTCTCAAAA
CGCACTGGCG ATGTGAACGG TCGCGGGATC GCGGGCGAGG AAAGCGAAAG AAGGGAAGGA AAGAGCGGTG CAAGCGGCCC GGAGAGTTTT

      4150       4160       4170       4180       4190       4200       4210       4220       4230
AAGGGAAAAA AAGCATGCAT CTCAATTAGT CAGCAACCAT AGTCCCGCCC CTAACTCCGC CCATCCCGCC CCTAACTCCG CCCAGTTCCG
TTCCCTTTTT TTCGTACGTA GAGTTAATCA GTCGTTGGTA TCAGGGCGGG GATTGAGGCG GGTAGGGCGG GGATTGAGGC GGGTCAAGGC

      4240       4250       4260       4270       4280       4290       4300       4310       4320
CCCATTCTCC GCCCCATGGC TGACTAATTT TTTTTATTTA TGCAGAGGCC GAGGCCGCCT CGGCCTCTGA GCTATTCCAG AAGTAGTGAG
GGGTAAGAGG CGGGGTACCG ACTGATTAAA AAAAATAAAT ACGTCTCCGG CTCCGGCGGA GCCGGAGACT CGATAAGGTC TTCATCACTC

      4330       4340       4350       4360       4370       4380       4390       4400       4410
GAGGCTTTTT TGGAGGCCTA GGCTTTTGCA AAAAGCTTGG ACAGCTCAGG GCTGCGATTT CGCGCCAAAC TTGACGGCAA TCCTAGCGTG
CTCCGAAAAA ACCTCCGGAT CCGAAAACGT TTTTCGAACC TGTCGAGTCC CGACGCTAAA GCGCGGTTTG AACTGCCGTT AGGATCGCAC

      4420       4430       4440       4450       4460       4470       4480       4490       4500
AAGGCTGGTA GGATTTTATC CCCGCTGCCA TCATGGTTCG ACCATTGAAC TGCATCGTCG CCGTGTCCCA AAATATGGGG ATTGGCAAGA
TTCCGACCAT CCTAAAATAG GGGCGACGGT AGTACCAAGC TGGTAACTTG ACGTAGCAGC GGCACAGGGT TTTATACCCC TAACCGTTCT
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Figure 14
(continued)

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      4510       4520       4530       4540       4550       4560       4570       4580       4590
ACGGAGACCT ACCCTGGCCT CCGCTCAGGA ACGAGTTCAA GTACTTCCAA AGAATGACCA CAACCTCTTC AGTGGAAGGT AAACAGAATC
TGCCTCTGGA TGGGACCGGA GGCGAGTCCT TGCTCAAGTT CATGAAGGTT TCTTACTGGT GTTGGAGAAG TCACCTTCCA TTTGTCTTAG

      4600       4610       4620       4630       4640       4650       4660       4670       4680
TGGTGATTAT GGGTAGGAAA ACCTGGTTCT CCATTCCTGA GAAGAATCGA CCTTTAAAGG ACAGAATTAA TATAGTTCTC AGTAGAGAAC
ACCACTAATA CCCATCCTTT TGGACCAAGA GGTAAGGACT CTTCTTAGCT GGAAATTTCC TGTCTTAATT ATATCAAGAG TCATCTCTTG

      4690       4700       4710       4720       4730       4740       4750       4760       4770
TCAAAGAACC ACCACGAGGA GCTCATTTTC TTGCCAAAAG TTTGGATGAT GCCTTAAGAC TTATTGAACA ACCGGAATTG GCAAGTAAAG
AGTTTCTTGG TGGTGCTCCT CGAGTAAAAG AACGGTTTTC AAACCTACTA CGGAATTCTG AATAACTTGT TGGCCTTAAC CGTTCATTTC

      4780       4790       4800       4810       4820       4830       4840       4850       4860
TAGACATGGT TTGGATAGTC GGAGGCAGTT CTGTTTACCA GGAAGCCATG AATCAACCAG GCCACCTTAG ACTCTTTGTG ACAAGGATCA
ATCTGTACCA AACCTATCAG CCTCCGTCAA GACAAATGGT CCTTCGGTAC TTAGTTGGTC CGGTGGAATC TGAGAAACAC TGTTCCTAGT

      4870       4880       4890       4900       4910       4920       4930       4940       4950
TGCAGGAATT TGAAAGTGAC ACGTTTTTCC CAGAAATTGA TTTGGGGAAA TATAAACTTC TCCCAGAATA CCCAGGCGTC CTCTCTGAGG
ACGTCCTTAA ACTTTCACTG TGCAAAAAGG GTCTTTAACT AAACCCCTTT ATATTTGAAG AGGGTCTTAT GGGTCCGCAG GAGAGACTCC

      4960       4970       4980       4990       5000       5010       5020       5030       5040
TCCAGGAGGA AAAAGGCATC AAGTATAAGT TTGAAGTCTA CGAGAAGAAA GACTAACAGG AAGATGCTTT CAAGTTCTCT GCTCCCCTCC
AGGTCCTCCT TTTTCCGTAG TTCATATTCA AACTTCAGAT GCTCTTCTTT CTGATTGTCC TTCTACGAAA GTTCAAGAGA CGAGGGGAGG

      5050       5060       5070       5080       5090       5100       5110       5120       5130
TAAAGCTATG CATTTTTATA AGACCATGGG ACTTTTGCTG GCTTTAGATC TCTTTGTGAA GGAACCTTAC TTCTGTGGTG TGACATAATT
ATTTCGATAC GTAAAAATAT TCTGGTACCC TGAAAACGAC CGAAATCTAG AGAAACACTT CCTTGGAATG AAGACACCAC ACTGTATTAA

      5140       5150       5160       5170       5180       5190       5200       5210       5220
GGACAAACTA CCTACAGAGA TTTAAAGCTC TAAGGTAAAT ATAAAATTTT TAAGTGTATA ATGTGTTAAA CTACTGATTC TAATTGTTTG
CCTGTTTGAT GGATGTCTCT AAATTTCGAG ATTCCATTTA TATTTTAAAA ATTCACATAT TACACAATTT GATGACTAAG ATTAACAAAC

      5230       5240       5250       5260       5270       5280       5290       5300       5310
TGTATTTTAG ATTCCAACCT ATGGAACTGA TGAATGGGAG CAGTGGTGGA ATGCCTTTAA TGAGGAAAAC CTGTTTTGCT CAGAAGAAAT
ACATAAAATC TAAGGTTGGA TACCTTGACT ACTTACCCTC GTCACCACCT TACGGAAATT ACTCCTTTTG GACAAAACGA GTCTTCTTTA

      5320       5330       5340       5350       5360       5370       5380       5390       5400
GCCATCTAGT GATGATGAGG CTACTGCTGA CTCTCAACAT TCTACTCCTC CAAAAAAGAA GAGAAAGGTA GAAGACCCCA AGGACTTTCC
CGGTAGATCA CTACTACTCC GATGACGACT GAGAGTTGTA AGATGAGGAG GTTTTTTCTT CTCTTTCCAT CTTCTGGGGT TCCTGAAAGG
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Figure 14
(continued)

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      5410       5420       5430       5440       5450       5460       5470       5480       5490
TTCAGAATTG CTAAGTTTTT TGAGTCATGC TGTGTTTAGT AATAGAACTC TTGCTTGCTT TGCTATTTAC ACCACAAAGG AAAAAGCTGC
AAGTCTTAAC GATTCAAAAA ACTCAGTACG ACACAAATCA TTATCTTGAG AACGAACGAA ACGATAAATG TGGTGTTTCC TTTTTCGACG

      5500       5510       5520       5530       5540       5550       5560       5570       5580
ACTGCTATAC AAGAAAATTA TGGAAAAATA TTCTGTAACC TTTATAAGTA GGCATAACAG TTATAATCAT AACATACTGT TTTTTCTTAC
TGACGATATG TTCTTTTAAT ACCTTTTTAT AAGACATTGG AAATATTCAT CCGTATTGTC AATATTAGTA TTGTATGACA AAAAAGAATG

      5590       5600       5610       5620       5630       5640       5650       5660       5670
TCCACACAGG CATAGAGTGT CTGCTATTAA TAACTATGCT CAAAAATTGT GTACCTTTAG CTTTTTAATT TGTAAAGGGG TTAATAAGGA
AGGTGTGTCC GTATCTCACA GACGATAATT ATTGATACGA GTTTTTAACA CATGGAAATC GAAAAATTAA ACATTTCCCC AATTATTCCT

      5680       5690       5700       5710       5720       5730       5740       5750       5760
ATATTTGATG TATAGTGCCT TGACTAGAGA TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC TTTAAAAAAC CTCCCACACC
TATAAACTAC ATATCACGGA ACTGATCTCT AGTATTAGTC GGTATGGTGT AAACATCTCC AAAATGAACG AAATTTTTTG GAGGGTGTGG

      5770       5780       5790       5800       5810       5820       5830       5840       5850
TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA
AGGGGGACTT GGACTTTGTA TTTTACTTAC GTTAACAACA ACAATTGAAC AAATAACGTC GAATATTACC AATGTTTATT TCGTTATCGT

      5860       5870       5880       5890       5900       5910       5920       5930       5940
TCACAAATTT CACAAATAAA GCATTTTTTT CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCG
AGTGTTTAAA GTGTTTATTT CGTAAAAAAA GTGACGTAAG ATCAACACCA AACAGGTTTG AGTAGTTACA TAGAATAGTA CAGACCTAGC

      5950       5960       5970       5980       5990       6000       6010       6020       6030
GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCAACTTGT TTATTGCAGC TTATAATGGT TACAAATAAA
CGACCTACTA GGAGGTCGCG CCCCTAGAGT ACGACCTCAA GAAGCGGGTG GGGTTGAACA AATAACGTCG AATATTACCA ATGTTTATTT

      6040       6050       6060       6070       6080       6090       6100       6110       6120
GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG
CGTTATCGTA GTGTTTAAAG TGTTTATTTC GTAAAAAAAG TGACGTAAGA TCAACACCAA ACAGGTTTGA GTAGTTACAT AGAATAGTAC

      6130       6140       6150       6160       6170       6180       6190       6200       6210
TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC ACAATTCCAC
AGACATATGG CAGCTGGAGA TCGATCTCGA ACCGCATTAG TACCAGTATC GACAAAGGAC ACACTTTAAC AATAGGCGAG TGTTAAGGTG

      6220       6230       6240       6250       6260       6270       6280       6290       6300
ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG
TGTTGTATGC TCGGCCTTCG TATTTCACAT TTCGGACCCC ACGGATTACT CACTCGATTG AGTGTAATTA ACGCAACGCG AGTGACGGGC
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Figure 14
(continued)

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      6310       6320       6330       6340       6350       6360       6370       6380       6390
CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG
GAAAGGTCAG CCCTTTGGAC AGCACGGTCG ACGTAATTAC TTAGCCGGTT GCGCGCCCCT CTCCGCCAAA CGCATAACCC GCGAGAAGGC

      6400       6410       6420       6430       6440       6450       6460       6470       6480
CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG
GAAGGAGCGA GTGACTGAGC GACGCGAGCC AGCAAGCCGA CGCCGCTCGC CATAGTCGAG TGAGTTTCCG CCATTATGCC AATAGGTGTC

      6490       6500       6510       6520       6530       6540       6550       6560       6570
AATCAGGGGA TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC
TTAGTCCCCT ATTGCGTCCT TTCTTGTACA CTCGTTTTCC GGTCGTTTTC CGGTCCTTGG CATTTTTCCG GCGCAACGAC CGCAAAAAGG

      6580       6590       6600       6610       6620       6630       6640       6650       6660
ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT
TATCCGAGGC GGGGGGACTG CTCGTAGTGT TTTTAGCTGC GAGTTCAGTC TCCACCGCTT TGGGCTGTCC TGATATTTCT ATGGTCCGCA

      6670       6680       6690       6700       6710       6720       6730       6740       6750
TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG
AAGGGGGACC TTCGAGGGAG CACGCGAGAG GACAAGGCTG GGACGGCGAA TGGCCTATGG ACAGGCGGAA AGAGGGAAGC CCTTCGCACC

      6760       6770       6780       6790       6800       6810       6820       6830       6840
CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC
GCGAAAGAGT TACGAGTGCG ACATCCATAG AGTCAAGCCA CATCCAGCAA GCGAGGTTCG ACCCGACACA CGTGCTTGGG GGGCAAGTCG

      6850       6860       6870       6880       6890       6900       6910       6920       6930
CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA
GGCTGGCGAC GCGGAATAGG CCATTGATAG CAGAACTCAG GTTGGGCCAT TCTGTGCTGA ATAGCGGTGA CCGTCGTCGG TGACCATTGT

      6940       6950       6960      .6970       6980       6990       7000       7010       7020
GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA
CCTAATCGTC TCGCTCCATA CATCCGCCAC GATGTCTCAA GAACTTCACC ACCGGATTGA TGCCGATGTG ATCTTCCTGT CATAAACCAT

      7030       7040       7050       7060       7070       7080       7090       7100       7110
TCTGCGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT
AGACGCGAGA CGACTTCGGT CAATGGAAGC CTTTTTCTCA ACCATCGAGA ACTAGGCCGT TTGTTTGGTG GCGACCATCG CCACCAAAAA

      7120       7130       7140       7150       7160       7170       7180       7190       7200
TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG
AACAAACGTT CGTCGTCTAA TGCGCGTCTT TTTTTCCTAG AGTTCTTCTA GGAAACTAGA AAAGATGCCC CAGACTGCGA GTCACCTTGC
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Figure 14
(continued)

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      7210       7220       7230       7240       7250       7260       7270       7280       7290
AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA
TTTTGAGTGC AATTCCCTAA AACCAGTACT CTAATAGTTT TTCCTAGAAG TGGATCTAGG AAAATTTAAT TTTTACTTCA AAATTTAGTT

      7300       7310       7320       7330       7340       7350       7360       7370       7380
TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT
AGATTTCATA TATACTCATT TGAACCAGAC TGTCAATGGT TACGAATTAG TCACTCCGTG GATAGAGTCG CTAGACAGAT AAAGCAAGTA

      7390       7400       7410       7420       7430       7440       7450       7460       7470
CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA CCGCGAGACC
GGTATCAACG GACTGAGGGG CAGCACATCT ATTGATGCTA TGCCCTCCCG AATGGTAGAC CGGGGTCACG ACGTTACTAT GGCGCTCTGG

      7480       7490       7500       7510       7520       7530       7540       7550       7560
CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA
GTGCGAGTGG CCGAGGTCTA AATAGTCGTT ATTTGGTCGG TCGGCCTTCC CGGCTCGCGT CTTCACCAGG ACGTTGAAAT AGGCGGAGGT

      7570       7580       7590       7600       7610       7620       7630       7640       7650
TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT ACAGGCATCG
AGGTCAGATA ATTAACAACG GCCCTTCGAT CTCATTCATC AAGCGGTCAA TTATCAAACG CGTTGCAACA ACGGTAACGA TGTCCGTAGC

      7660       7670       7680       7690       7700       7710       7720       7730       7740
TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA
ACCACAGTGC GAGCAGCAAA CCATACCGAA GTAAGTCGAG GCCAAGGGTT GCTAGTTCCG CTCAATGTAC TAGGGGGTAC AACACGTTTT

      7750       7760       7770       7780       7790       7800       7810       7820       7830
AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT
TTCGCCAATC GAGGAAGCCA GGAGGCTAGC AACAGTCTTC ATTCAACCGG CGTCACAATA GTGAGTACCA ATACCGTCGT GACGTATTAA

      7840       7850       7860       7870       7880       7890       7900       7910       7920
CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA
GAGAATGACA GTACGGTAGG CATTCTACGA AAAGACACTG ACCACTCATG AGTTGGTTCA GTAAGACTCT TATCACATAC GCCGCTGGCT

      7930       7940       7950       7960       7970       7980       7990       8000       8010
GTTGCTCTTG CCCGGCGTCA ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC
CAACGAGAAC GGGCCGCAGT TATGCCCTAT TATGGCGCGG TGTATCGTCT TGAAATTTTC ACGAGTAGTA ACCTTTTGCA AGAAGCCCCG

      8020       8030       8040       8050       8060       8070       8080       8090       8100
GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA
CTTTTGAGAG TTCCTAGAAT GGCGACAACT CTAGGTCAAG CTACATTGGG TGAGCACGTG GGTTGACTAG AAGTCGTAGA AAATGAAAGT
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Figure 14
(continued)

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      8110       8120       8130       8140       8150       8160       8170       8180       8190
CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT
GGTCGCAAAG ACCCACTCGT TTTTGTCCTT CCGTTTTACG GCGTTTTTTC CCTTATTCCC GCTGTGCCTT TACAACTTAT GAGTATGAGA

      8200       8210       8220       8230       8240       8250       8260       8270       8280
TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG
AGGAAAAAGT TATAATAACT TCGTAAATAG TCCCAATAAC AGAGTACTCG CCTATGTATA AACTTACATA AATCTTTTTA TTTGTTTATC

      8290       8300       8310       8320       8330
GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT C
CCCAAGGCGC GTGTAAAGGG GCTTTTCACG GTGGACTGCA G
```

Figure 14
(continued)

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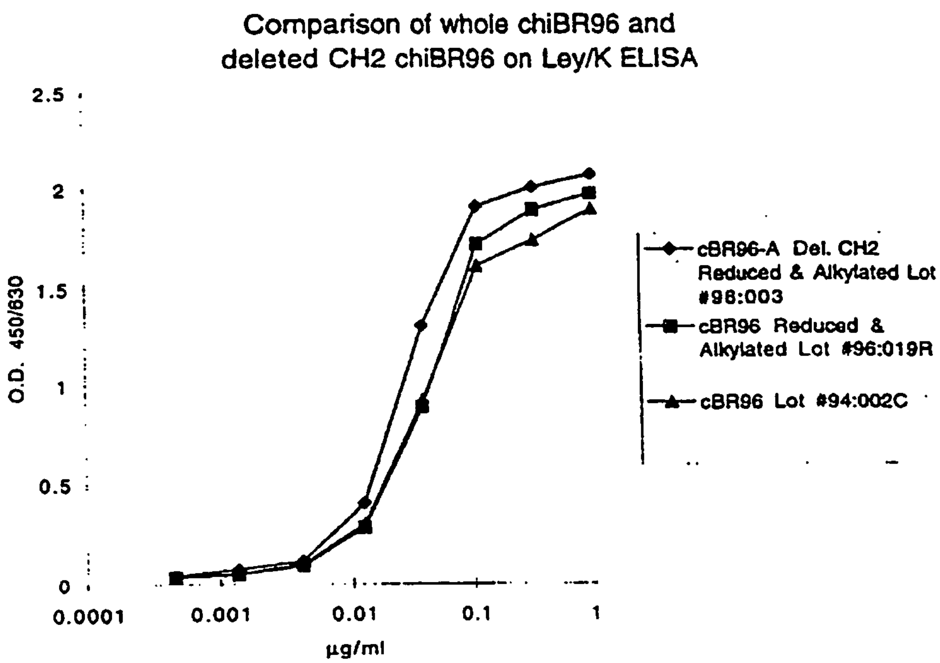
Comparison of whole chiBR96 and
deleted CH2 chiBR96 on Ley/K ELISA
O.D. 450/630
2.5
2
1.5
1
0.5
0
0.0001
0.001
0.01
0.1
1
µg/ml
cBR96-A Del. CH2 Reduced & Alkylated Lot #96:003
cBR96 Reduced & Alkylated Lot #96:019R
cBR96 Lot #94:002C

Figure 15

hBR96-2B: L235 to A235 and G237 to A237

hBR96-2C: E318 to S318, K320 to S320, and K322 to S322

hBR96-2D: P331 to A331

hBR96-2E: L235 to A235, G237 to A237, E318 to S318, K320 to S320, and K322 to S322

hBR96-2F: L235 to A235, G237 to A237, and P331 to A331

hBR96-2G: E318 to S318, K320 to S320, K322 to S322, and P331 to A331

hBR96-2H: L235 to A235, G237 to A237, E318 to S318, K320 to S320, K322 to S322, and P331 to A331

Figure 16

FIGURE 17

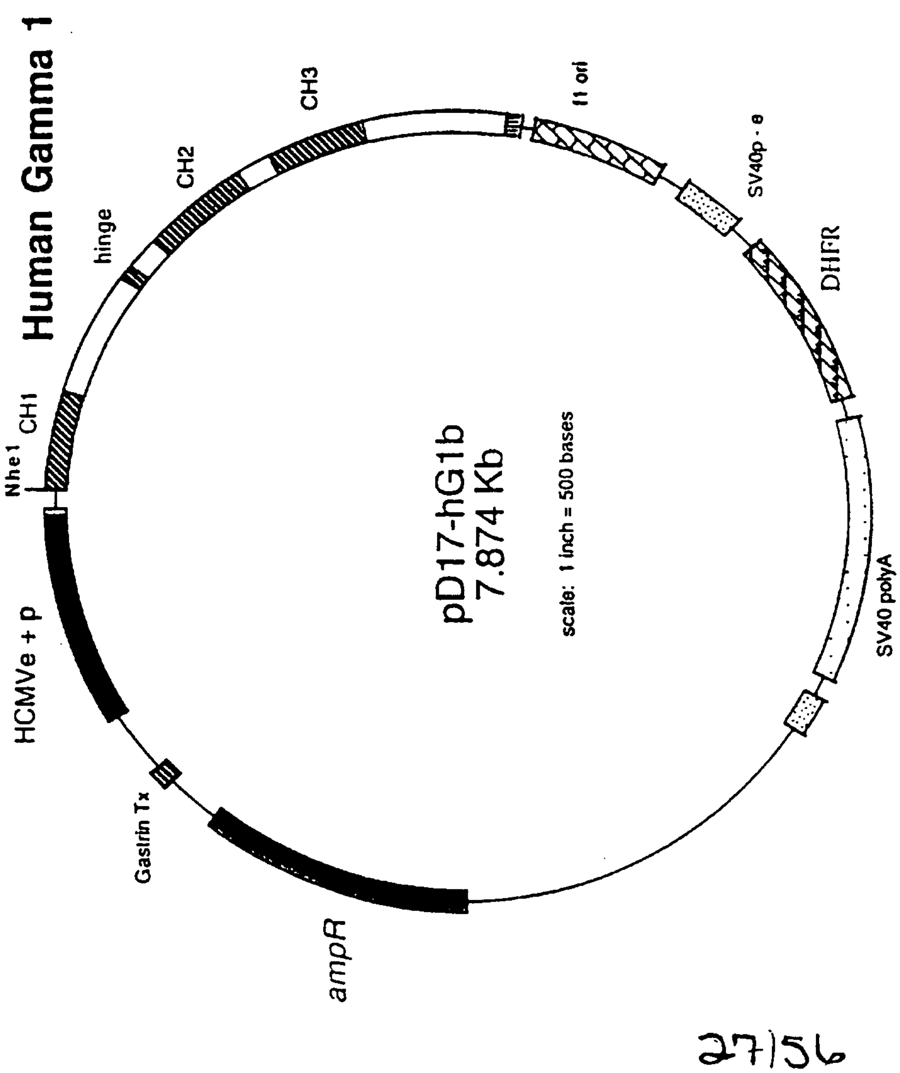
Human Gamma 1
pD17-hG1b
7.874 Kb
scale: 1 inch = 500 bases
Nhe1
CH1
hinge
CH2
CH3
f1 ori
SV40p - e
DHFR
SV40 polyA
ampR
Gastrin Tx
HCMVe + p

FIGURE 18A

1 GGTACCAATT TAAATTGATA TCTCCTTAGG TCTCGAGTCT CTAGATAACC
51 GGTCAATCGA TTGGAATTCT TGCGGCCGCT TGCTAGCCAC CATGGAGTTG
101 TGGTTAAGCT TGGTCTTCCT TGTCCTTGTT TTAAAAGGTG TCCAGTGTGA
151 AGTGCAACTG GTGGAGTCTG GGGGAGGCTT AGTGCAGCCT GGAGGGTCCC
201 TGCGACTTTC CTGTGCTGCA TCTGGATTCC CGTTCAGTGA CTATTACATG
251 TATTGGGTTC GCCAGGCTCC AGGCAAGGGA CTGGAGTGGG TCTCATACAT
301 TAGTCAAGAT GGTGATATAA CCGACTATGC AGACTCCGTA AAGGGTCGAT
351 TCACCATCTC CAGAGACAAT GCAAAGAACA GCCTGTACCT GCAAATGAAC
401 AGCCTGAGGG ACGAGGACAC AGCCGTGTAT TACTGTGCAA GAGGCCTGGC
451 GGACGGGGCC TGGTTTGCTT ACTGGGGCCA AGGGACTCTG GTCACGGTCT
501 CTTCCGCTAG CACCAAGGGC CCATCGGTCT TCCCCCTGGC ACCCTCCTCC
551 AAGAGCACCT CTGGGGGCAC AGCGGCCCTG GGCTGCCTGG TCAAGGACTA
601 CTTCCCCGAA CCGGTGACGG TGTCGTGGAA CTCAGGCGCC CTGACCAGCG
651 GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC
701 AGCAGCGTGG TCACCGTGCC CTCCAGCAGC TTGGGCACCC AGACCTACAT
751 CTGCAACGTG AATCACAAGC CCAGCAACAC CAAGGTGGAC AAGAAAGTTG
801 GTGAGAGGCC AGCACAGGGA GGGAGGGTGT CTGCTGGAAG CCAGGCTCAG
851 CGCTCCTGCC TGGACGCATC CCGGCTATGC AGCCCCAGTC CAGGGCAGCA
901 AGGCAGGCCC CGTCTGCCTC TTCACCCGGA GGCCTCTGCC CGCCCCACTC
951 ATGCTCAGGG AGAGGGTCTT CTGGCTTTTT CCCCAGGCTC TGGGCAGGCA
1001 CAGGCTAGGT GCCCCTAACC CAGGCCCTGC ACACAAAGGG GCAGGTGCTG
1051 GGCTCAGACC TGCCAAGAGC CATATCCGGG AGGACCCTGC CCCTGACCTA
1101 AGCCCACCCC AAAGGCCAAA CTCTCCACTC CCTCAGCTCG GACACCTTCT
1151 CTCCTCCCAG ATTCCAGTAA CTCCCAATCT TCTCTCTGCA GAGCCCAAAT
1201 CTTGTGACAA AACTCACACA TGCCCACCGT GCCCAGGTAA GCCAGCCCAG
1251 GCCTCGCCCT CCAGCTCAAG GCGGGACAGG TGCCCTAGAG TAGCCTGCAT
1301 CCAGGGACAG GCCCCAGCCG GGTGCTGACA CGTCCACCTC CATCTCTTCC

235 237

1351 TCAGCACCTG AACTCCTGGG GGGACCGTCA GTCTTCCTCT TCCCCCCAAA

1401 ACCCAAGGAC ACCCTCATGA TCTCCCGGAC CCCTGAGGTC ACATGCGTGG

1451 TGGTGGACGT GAGCCACGAA GACCCTGAGG TCAAGTTCAA CTGGTACGTG

1501 GACGGCGTGG AGGTGCATAA TGCCAAGACA AAGCCGCGGG AGGAGCAGTA

1551 CAACAGCACG TACCGTGTGG TCAGCGTCCT CACCGTCCTG CACCAGGACT

318 320 322

1601 GGCTGAATGG CAAGGAGTAC AAGTGCAAGG TCTCCAACAA AGCCCTCCCA

331

1651 GCCCCCATCG AGAAAACCAT CTCCAAAGCC AAAGGTGGGA CCCGTGGGGT

1701 GCGAGGGCCA CATGGACAGA GGCCGGCTCG GCCCACCCTC TGCCCTGAGA

1751 GTGACCGCTG TACCAACCTC TGTCCCTACA GGGCAGCCCC GAGAACCACA

1801 GGTGTACACC CTGCCCCCAT CCCGGGATGA GCTGACCAAG AACCAGGTCA

1851 GCCTGACCTG CCTGGTCAAA GGCTTCTATC CCAGCGACAT CGCCGTGGAG

1901 TGGGAGAGCA ATGGGCAGCC GGAGAACAAC TACAAGACCA CGCCTCCCGT

1951 GCTGGACTCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC ACCGTGGACA

2001 AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG

2051 GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCCCTGT CTCCGGGTAA

2101 ATGAGTGCGA CGGCCGGCAA GCCCCCGCTC CCCGGGCTCT CGCGGTCGCA

2151 CGAGGATGCT TGGCACGTAC CCCCTGTACA TACTTCCCGG GCGCCCAGCA

2201 TGGAAATAAA GCACCCAGCG CTGCCCTGGG CCCCTGCGAG ACTGTGATGG

2251 TTCTTTCCAC GGGTCAGGCC GAGTCTGAGG CCTGAGTGGC ATGAGGGAGG

2301 CAGAGCGGGT CCCACTGTCC CCACACTGGC CCAGGCTGTG CAGGTGTGCC

2351 TGGGCCCCCT AGGGTGGGGC TCAGCCAGGG GCTGCCCTCG GCAGGGTGGG

2401 GGATTTGCCA GCGTGGCCCT CCCTCCAGCA GCACCTGCCC TGGGCTGGGC

2451 CACGGGAAGC CCTAGGAGCC CCTGGGGACA GACACACAGC CCCTGCCTCT

2501 GTAGGAGACT GTCCTGTTCT GTGAGCGCCC CTGTCCTCCC GACCTCCATG

2551 CCCACTCGGG GGCATGCCTA GTCCATGTGC GTAGGGACAG GCCCTCCCTC

2601 ACCCATCTAC CCCCACGGCA CTAACCCCTG GCTGCCCTGC CCAGCCTCGC

2651 ACCCGCATGG GGACACAACC GACTCCGGGG ACATGCACTC TCGGGCCCTG

2701 TGGAGGGACT GGTGCAGATG CCCACACACA CACTCAGCCC AGACCCGTTC

2751 AACAAACCCC GCACTGAGGT TGGCCGGCCA CACGGCCACC ACACACACAC

2801 GTGCACGCCT CACACACGGA GCCTCACCCG GGCGAACTGC ACAGCACCCA

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FIGURE 18B

2851 GACCAGAGCA AGGACCTCGC ACACGTGAAC ACTCCTCGGA CACAGGCCCC
2901 CACGAGCCCC ACGCGGCACC TCAAGGCCCA CGAGCCTCTC GGCAGCTTCT
2951 CCACATGCTG ACCTGCTCAG ACAAACCCAG CCCTCCTCTC ACAAGGGTGC
3001 CCCTGCAGCC GCCACACACA CACAGGGGAT CACACACCAC GTCACGTCCC
3051 TGGCCCTGGC CCACTTCCCA GTGCCGCCCT TCCCTGCAGG ACGGATCAGC
3101 CTCGACTGTG CCTTCTAGTT GCCAGCCATC TGTTGTTTGC CCCTCCCCCG
3151 TGCCTTCCTT GACCCTGGAA GGTGCCACTC CCACTGTCCT TTCCTAATAA
3201 AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGTCATT CTATTCTGGG
3251 GGGTGGGGTG GGGCAGGACA GCAAGGGGGA GGATTGGGAA GACAATAGCA
3301 GGCATGCTGG GGATGCGGTG GGCTCTATGG CTTCTGAGGC GGAAAGAACC
3351 AGCTGGGGCT CTAGGGGGTA TCCCCACGCG CCCTGTAGCG GCGCATTAAG
3401 CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA CTTGCCAGCG
3451 CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC
3501 GCCGGGCCTC TCAAAAAAGG GAAAAAAAGC ATGCATCTCA ATTAGTCAGC
3551 AACCATAGTC CCGCCCCTAA CTCCGCCCAT CCCGCCCCTA ACTCCGCCCA
3601 GTTCCGCCCA TTCTCCGCCC CATGGCTGAC TAATTTTTTT TATTTATGCA
3651 GAGGCCGAGG CCGCCTCGGC CTCTGAGCTA TTCCAGAAGT AGTGAGGAGG
3701 CTTTTTTGGA GGCCTAGGCT TTTGCAAAAA GCTTGGACAG CTCAGGGCTG
3751 CGATTTCGCG CCAAACTTGA CGGCAATCCT AGCGTGAAGG CTGGTAGGAT
3801 TTTATCCCCG CTGCCATCAT GGTTCGACCA TTGAACTGCA TCGTCGCCGT
3851 GTCCCAAAAT ATGGGGATTG GCAAGAACGG AGACCTACCC TGGCCTCCGC
3901 TCAGGAACGA GTTCAAGTAC TTCCAAAGAA TGACCACAAC CTCTTCAGTG
3951 GAAGGTAAAC AGAATCTGGT GATTATGGGT AGGAAAACCT GGTTCTCCAT
4001 TCCTGAGAAG AATCGACCTT TAAAGGACAG AATTAATATA GTTCTCAGTA
4051 GAGAACTCAA AGAACCACCA CGAGGAGCTC ATTTTCTTGC CAAAAGTTTG
4101 GATGATGCCT TAAGACTTAT TGAACAACCG GAATTGGCAA GTAAAGTAGA
4151 CATGGTTTGG ATAGTCGGAG GCAGTTCTGT TTACCAGGAA GCCATGAATC
4201 AACCAGGCCA CCTTAGACTC TTTGTGACAA GGATCATGCA GGAATTTGAA
4251 AGTGACACGT TTTTCCCAGA AATTGATTTG GGGAAATATA AACTTCTCCC
4301 AGAATACCCA GGCGTCCTCT CTGAGGTCCA GGAGGAAAAA GGCATCAAGT

FIGURE 18C

4351 ATAAGTTTGA AGTCTACGAG AAGAAAGACT AACAGGAAGA TGCTTTCAAG
4401 TTCTCTGCTC CCCTCCTAAA GCTATGCATT TTTATAAGAC CATGGGACTT
4451 TTGCTGGCTT TAGATCTCTT TGTGAAGGAA CCTTACTTCT GTGGTGTGAC
4501 ATAATTGGAC AAACTACCTA CAGAGATTTA AAGCTCTAAG GTAAATATAA
4551 AATTTTTAAG TGTATAATGT GTTAAACTAC TGATTCTAAT TGTTTGTGTA
4601 TTTTAGATTC CAACCTATGG AACTGATGAA TGGGAGCAGT GGTGGAATGC
4651 CTTTAATGAG GAAAACCTGT TTTGCTCAGA AGAAATGCCA TCTAGTGATG
4701 ATGAGGCTAC TGCTGACTCT CAACATTCTA CTCCTCCAAA AAAGAAGAGA
4751 AAGGTAGAAG ACCCCAAGGA CTTTCCTTCA GAATTGCTAA GTTTTTTGAG
4801 TCATGCTGTG TTTAGTAATA GAACTCTTGC TTGCTTTGCT ATTTACACCA
4851 CAAAGGAAAA AGCTGCACTG CTATACAAGA AAATTATGGA AAAATATTCT
4901 GTAACCTTTA TAAGTAGGCA TAACAGTTAT AATCATAACA TACTGTTTTT
4951 TCTTACTCCA CACAGGCATA GAGTGTCTGC TATTAATAAC TATGCTCAAA
5001 AATTGTGTAC CTTTAGCTTT TTAATTTGTA AAGGGGTTAA TAAGGAATAT
5051 TTGATGTATA GTGCCTTGAC TAGAGATCAT AATCAGCCAT ACCACATTTG
5101 TAGAGGTTTT ACTTGCTTTA AAAAACCTCC CACACCTCCC CCTGAACCTG
5151 AAACATAAAA TGAATGCAAT TGTTGTTGTT AACTTGTTTA TTGCAGCTTA
5201 TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT
5251 TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT
5301 TATCATGTCT GGATCGGCTG GATGATCCTC CAGCGCGGGG ATCTCATGCT
5351 GGAGTTCTTC GCCCACCCCA ACTTGTTTAT TGCAGCTTAT AATGGTTACA
5401 AATAAAGCAA TAGCATCACA AATTTCACAA ATAAAGCATT TTTTTCACTG
5451 CATTCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG
5501 TATACCGTCG ACCTCTAGCT AGAGCTTGGC GTAATCATGG TCATAGCTGT
5551 TTCCTGTGTG AAATTGTTAT CCGCTCACAA TTCCACACAA CATACGAGCC
5601 GGAAGCATAA AGTGTAAAGC CTGGGGTGCC TAATGAGTGA GCTAACTCAC
5651 ATTAATTGCG TTGCGCTCAC TGCCCGCTTT CCAGTCGGGA AACCTGTCGT
5701 GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG CGGTTTGCGT
5751 ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT
5801 TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT

FIGURE 18D

```
5851  CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG
5901  CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG
5951  GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT
6001  GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC
6051  TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC
6101  CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCAATGC TCACGCTGTA
6151  GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC
6201  GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT
6251  TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG
6301  GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG
6351  AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG
6401  CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT
6451  CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG
6501  CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC
6551  TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG
6601  TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA
6651  TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG
6701  TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC
6751  GTTCATCCAT AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG
6801  GAGGGCTTAC CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG
6851  CTCACCGGCT CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG
6901  AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT
6951  TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA
7001  CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA
7051  TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC
7101  CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT
7151  CAGAAGTAAG TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC
7201  ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT
7251  GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG
7301  CTCTTGCCCG GCGTCAATAC GGGATAATAC CGCGCCACAT AGCAGAACTT
```

FIGURE 18E

7351 TAAAAGTGCT CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG
7401 ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA
7451 CTGATCTTCA GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA
7501 CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT
7551 TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG
7601 TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC
7651 AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCGAC
7701 GGATCGGGAG ATCTGCTAGG TGACCTGAGG CGCGCCGGCT TCGAATAGCC
7751 AGAGTAACCT TTTTTTTTAA TTTTATTTTA TTTTATTTTT GAGATGGAGT
7801 TTGGCGCCGA TCTCCCGATC CCCTATGGTC GACTCTCAGT ACAATCTGCT
7851 CTGATGCCGC ATAGTTAAGC CAGTATCTGC TCCCTGCTTG TGTGTTGGAG
7901 GTCGCTGAGT AGTGCGCGAG CAAAATTTAA GCTACAACAA GGCAAGGCTT
7951 GACCGACAAT TGCATGAAGA ATCTGCTTAG GGTTAGGCGT TTTGCGCTGC
8001 TTCGCGATGT ACGGGCCAGA TATACGCGTT GACATTGATT ATTGACTAGT
8051 TATTAATAGT AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA
8101 GTTCCGCGTT ACATAACTTA CGGTAAATGG CCCGCCTGGC TGACCGCCCA
8151 ACGACCCCCG CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG
8201 CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGACTATT TACGGTAAAC
8251 TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA
8301 TTGACGTCAA TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG
8351 ACCTTATGGG ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC
8401 TATTACCATG GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC
8451 GGTTTGACTC ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG
8501 AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA
8551 CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT
8601 ATATAAGCAG AGCTCTCTGG CTAACTAGAG AACCCACTGC TTACTGGCTT
8651 ATCGAAATTA ATACGACTCA CTATAGGGAG ACCCAAGCTT

FIGURE 18F

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FIGURE 19 A

pD17-hG1b

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        10         20         30         40         50         60
GGTACCAATT TAAATTGATA TCTCCTTAGG TCTCGAGTCT CTAGATAACC GGTCAATCGA
CCATGGTTAA ATTTAACTAT AGAGGAATCC AGAGCTCAGA GATCTATTGG CCAGTTAGCT

        70         80         90        100        110        120
TTGGAATTCT TGCGGCCGCT TGCTAGCACC AAGGGCCCAT CGGTCTTCCC CCTGGCACCC
AACCTTAAGA ACGCCGGCGA ACGATCGTGG TTCCCGGGTA GCCAGAAGGG GGACCGTGGG

       130        140        150        160        170        180
TCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT GCCTGGTCAA GGACTACTTC
AGGAGGTTCT CGTGGAGACC CCCGTGTCGC CGGGACCCGA CGGACCAGTT CCTGATGAAG

       190        200        210        220        230        240
CCCGAACCGG TGACGGTGTC GTGGAACTCA GGCGCCCTGA CCAGCGGCGT GCACACCTTC
GGGCTTGGCC ACTGCCACAG CACCTTGAGT CCGCGGGACT GGTCGCCGCA CGTGTGGAAG

       250        260        270        280        290        300
CCGGCTGTCC TACAGTCCTC AGGACTCTAC TCCCTCAGCA GCGTGGTCAC CGTGCCCTCC
GGCCGACAGG ATGTCAGGAG TCCTGAGATG AGGGAGTCGT CGCACCAGTG GCACGGGAGG

       310        320        330        340        350        360
AGCAGCTTGG GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCCAG CAACACCAAG
TCGTCGAACC CGTGGGTCTG GATGTAGACG TTGCACTTAG TGTTCGGGTC GTTGTGGTTC

       370        380        390        400        410        420
GTGGACAAGA AAGTTGGTGA GAGGCCAGCA CAGGGAGGGA GGGTGTCTGC TGGAAGCCAG
CACCTGTTCT TTCAACCACT CTCCGGTCGT GTCCCTCCCT CCCACAGACG ACCTTCGGTC

       430        440        450        460        470        480
GCTCAGCGCT CCTGCCTGGA CGCATCCCGG CTATGCAGCC CCAGTCCAGG GCAGCAAGGC
CGAGTCGCGA GGACGGACCT GCGTAGGGCC GATACGTCGG GGTCAGGTCC CGTCGTTCCG

       490        500        510        520        530        540
AGGCCCCGTC TGCCTCTTCA CCCGGAGGCC TCTGCCCGCC CCACTCATGC TCAGGGAGAG
TCCGGGGCAG ACGGAGAAGT GGGCCTCCGG AGACGGGCGG GGTGAGTACG AGTCCCTCTC

       550        560        570        580        590        600
GGTCTTCTGG CTTTTTCCCC AGGCTCTGGG CAGGCACAGG CTAGGTGCCC CTAACCCAGG
CCAGAAGACC GAAAAAGGGG TCCGAGACCC GTCCGTGTCC GATCCACGGG GATTGGGTCC
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FIGURE 19B

pD17-hG1b

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       610        620        630        640        650        660
CCCTGCACAC AAAGGGGCAG GTGCTGGGCT CAGACCTGCC AAGAGCCATA TCCGGGAGGA
GGGACGTGTG TTTCCCCGTC CACGACCCGA GTCTGGACGG TTCTCGGTAT AGGCCCTCCT

       670        680        690        700        710        720
CCCTGCCCCT GACCTAAGCC CACCCCAAAG GCCAAACTCT CCACTCCCTC AGCTCGGACA
GGGACGGGGA CTGGATTCGG GTGGGGTTTC CGGTTTGAGA GGTGAGGGAG TCGAGCCTGT

       730        740        750        760        770        780
CCTTCTCTCC TCCCAGATTC CAGTAACTCC CAATCTTCTC TCTGCAGAGC CCAAATCTTG
GGAAGAGAGG AGGGTCTAAG GTCATTGAGG GTTAGAAGAG AGACGTCTCG GGTTTAGAAC

       790        800        810        820        830        840
TGACAAAACT CACACATGCC CACCGTGCCC AGGTAAGCCA GCCCAGGCCT CGCCCTCCAG
ACTGTTTTGA GTGTGTACGG GTGGCACGGG TCCATTCGGT CGGGTCCGGA GCGGGAGGTC

       850        860        870        880        890        900
CTCAAGGCGG GACAGGTGCC CTAGAGTAGC CTGCATCCAG GGACAGGCCC CAGCCGGGTG
GAGTTCCGCC CTGTCCACGG GATCTCATCG GACGTAGGTC CCTGTCCGGG GTCGGCCCAC

       910        920        930        940  235   950 237    960
CTGACACGTC CACCTCCATC TCTTCCTCAG CACCTGAACT CCTGGGGGGA CCGTCAGTCT
GACTGTGCAG GTGGAGGTAG AGAAGGAGTC GTGGACTTGA GGACCCCCCT GGCAGTCAGA

       970        980        990       1000       1010       1020
TCCTCTTCCC CCCAAAACCC AAGGACACCC TCATGATCTC CCGGACCCCT GAGGTCACAT
AGGAGAAGGG GGGTTTTGGG TTCCTGTGGG AGTACTAGAG GGCCTGGGGA CTCCAGTGTA

      1030       1040       1050       1060       1070       1080
GCGTGGTGGT GGACGTGAGC CACGAAGACC CTGAGGTCAA GTTCAACTGG TACGTGGACG
CGCACCACCA CCTGCACTCG GTGCTTCTGG GACTCCAGTT CAAGTTGACC ATGCACCTGC

      1090       1100       1110       1120       1130       1140
GCGTGGAGGT GCATAATGCC AAGACAAAGC CGCGGGAGGA GCAGTACAAC AGCACGTACC
CGCACCTCCA CGTATTACGG TTCTGTTTCG GCGCCCTCCT CGTCATGTTG TCGTGCATGG

                                                     318   320
      1150       1160       1170       1180       1190       1200
CTCTGGTCAG CGTCCTCACC GTCCTGCACC AGGACTGGCT GAATGGCAAG GAGTACAAGT
CACACCAGTC GCAGGAGTGG CAGGACGTGG TCCTGACCCA CTTACCGTTC CTCATGTTCA
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FIGURE 19C

pD17-hG1b

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322     1210       1220       1230 331   1240       1250       1260
GCAAGGTCTC CAACAAAGCC CTCCCAGCCC CCATCGAGAA AACCATCTCC AAAGCCAAAG
CGTTCCAGAG GTTGTTTCGG GAGGGTCGGG GGTAGCTCTT TTGGTAGAGG TTTCGGTTTC

      1270       1280       1290       1300       1310       1320
GTGGGACCCG TGGGGTGCGA GGGCCACATG GACAGAGGCC GGCTCGGCCC ACCCTCTGCC
CACCCTGGGC ACCCCACGCT CCCGGTGTAC CTGTCTCCGG CCGAGCCGGG TGGGAGACGG

      1330       1340       1350       1360       1370       1380
CTGAGAGTGA CCGCTGTACC AACCTCTGTC CCTACAGGGC AGCCCCGAGA ACCACAGGTG
GACTCTCACT GGCGACATGG TTGGAGACAG GGATGTCCCG TCGGGGCTCT TGGTGTCCAC

      1390       1400       1410       1420       1430       1440
TACACCCTGC CCCCATCCCG GGATGAGCTG ACCAAGAACC AGGTCAGCCT GACCTGCCTG
ATGTGGGACG GGGGTAGGGC CCTACTCGAC TGGTTCTTGG TCCAGTCGGA CTGGACGGAC

      1450       1460       1470       1480       1490       1500
GTCAAAGGCT TCTATCCCAG CGACATCGCC GTGGAGTGGG AGAGCAATGG GCAGCCGGAG
CAGTTTCCGA AGATAGGGTC GCTGTAGCGG CACCTCACCC TCTCGTTACC CGTCGGCCTC

      1510       1520       1530       1540       1550       1560
AACAACTACA AGACCACGCC TCCCGTGCTG GACTCCGACG GCTCCTTCTT CCTCTACAGC
TTGTTGATGT TCTGGTGCGG AGGGCACGAC CTGAGGCTGC CGAGGAAGAA GGAGATGTCG

      1570       1580       1590       1600       1610       1620
AAGCTCACCG TGGACAAGAG CAGGTGGCAG CAGGGGAACG TCTTCTCATG CTCCGTGATG
TTCGAGTGGC ACCTGTTCTC GTCCACCGTC GTCCCCTTGC AGAAGAGTAC GAGGCACTAC

      1630       1640       1650       1660       1670       1680
CATGAGGCTC TGCACAACCA CTACACGCAG AAGAGCCTCT CCCTGTCTCC GGGTAAATGA
GTACTCCGAG ACGTGTTGGT GATGTGCGTC TTCTCGGAGA GGGACAGAGG CCCATTTACT

      1690       1700       1710       1720       1730       1740
GTGCGACGGC CGGCAAGCCC CCGCTCCCCG GGCTCTCGCG GTCGCACGAG GATGCTTGGC
CACGCTGCCG GCCGTTCGGG GGCGAGGGGC CCGAGAGCGC CAGCGTGCTC CTACGAACCG

      1750       1760       1770       1780       1790       1800
ACGTACCCCC TGTACATACT TCCCGGGCGC CCAGCATGGA AATAAAGCAC CCAGCGCTGC
TGCATGGGGG ACATGTATGA AGGGCCCGCG GGTCGTACCT TTATTTCGTG GGTCGCGACG
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FIGURE 19D

pD17-hG1b

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      1810       1820       1830       1840       1850       1860
CCTGGGCCCC TGCGAGACTG TGATGGTTCT TTCCACGGGT CAGGCCGAGT CTGAGGCCTG
GGACCCGGGG ACGCTCTGAC ACTACCAAGA AAGGTGCCCA GTCCGGCTCA GACTCCGGAC

      1870       1880       1890       1900       1910       1920
AGTGGCATGA GGGAGGCAGA GCGGGTCCCA CTGTCCCCAC ACTGGCCCAG GCTGTGCAGG
TCACCGTACT CCCTCCGTCT CGCCCAGGGT GACAGGGGTG TGACCGGGTC CGACACGTCC

      1930       1940       1950       1960       1970       1980
TGTGCCTGGG CCCCCTAGGG TGGGGCTCAG CCAGGGGCTG CCCTCGGCAG GGTGGGGGAT
ACACGGACCC GGGGGATCCC ACCCCGAGTC GGTCCCCGAC GGGAGCCGTC CCACCCCCTA

      1990       2000       2010       2020       2030       2040
TTGCCAGCGT GGCCCTCCCT CCAGCAGCAC CTGCCCTGGG CTGGGCCACG GGAAGCCCTA
AACGGTCGCA CCGGGAGGGA GGTCGTCGTG GACGGGACCC GACCCGGTGC CCTTCGGGAT

      2050       2060       2070       2080       2090       2100
GGAGCCCCTG GGGACAGACA CACAGCCCCT GCCTCTGTAG GAGACTGTCC TGTTCTGTGA
CCTCGGGGAC CCCTGTCTGT GTGTCGGGGA CGGAGACATC CTCTGACAGG ACAAGACACT

      2110       2120       2130       2140       2150       2160
GCGCCCCTGT CCTCCCGACC TCCATGCCCA CTCGGGGGCA TGCTGGGGAT GCGGTGGGCT
CGCGGGGACA GGAGGGCTGG AGGTACGGGT GAGCCCCCGT ACGACCCCTA CGCCACCCGA

      2170       2180       2190       2200       2210       2220
CTATGGCTTC TGAGGCGGAA AGAACCAGCT GGGGCTCTAG GGGTATCCC CACGCGCCCT
GATACCGAAG ACTCCGCCTT TCTTGGTCGA CCCCGAGATC CCCCATAGGG GTGCGCGGGA

      2230       2240       2250       2260       2270       2280
GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG
CATCGCCGCG TAATTCGCGC CGCCCACACC ACCAATGCGC GTCGCACTGG CGATGTGAAC

      2290       2300       2310       2320       2330       2340
CCAGCGCCCT AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG
GGTCGCGGGA TCGCGGGCGA GGAAAGCGAA AGAAGGGAAG GAAAGAGCGG TGCAAGCGGC

      2350       2360       2370       2380       2390       2400
GCTTTCCCCG TCAAGCTCTA AATCGGGGCA TCCCTTTAGG GTTCCGATTT AGTGCTTTAC
CGAAAGGGGC AGTTCGAGAT TTAGCCCCGT AGGGAAATCC CAAGGCTAAA TCACGAAATG
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FIGURE 19E

pD17-hG1b

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      2410       2420       2430       2440       2450       2460
GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG CCATCGCCCT
CCGTGGAGCT GGGGTTTTTT GAACTAATCC CACTACCAAG TGCATCACCC GGTAGCGGGA

      2470       2480       2490       2500       2510       2520
GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTTAATAGT GGACTCTTGT
CTATCTGCCA AAAAGCGGGA AACTGCAACC TCAGGTGCAA GAAATTATCA CCTGAGAACA

      2530       2540       2550       2560       2570       2580
TCCAAACTGG AACAACACTC AACCCTATCT CGGTCTATTC TTTTGATTTA TAAGGGATTT
AGGTTTGACC TTGTTGTGAG TTGGGATAGA GCCAGATAAG AAAACTAAAT ATTCCCTAAA

      2590       2600       2610       2620       2630       2640
TGGGGATTTC GGCCTATTGG TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT
ACCCCTAAAG CCGGATAACC AATTTTTTAC TCGACTAAAT TGTTTTTAAA TTGCGCTTAA

      2650       2660       2670       2680       2690       2700
AATTCTGTGG AATGTGTGTC AGTTAGGGTG TGGAAAGTCC CCAGGCTCCC CAGGCAGGCA
TTAAGACACC TTACACACAG TCAATCCCAC ACCTTTCAGG GGTCCGAGGG GTCCGTCCGT

      2710       2720       2730       2740       2750       2760
GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAT AGTCCCGCCC CTAACTCCGC
CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTA TCAGGGCGGG GATTGAGGCG

      2770       2780       2790       2800       2810       2820
CCATCCCGCC CCTAACTCCG CCCAGTTCCG CCCATTCTCC GCCCCATGGC TGACTAATTT
GGTAGGGCGG GGATTGAGGC GGGTCAAGGC GGGTAAGAGG CGGGGTACCG ACTGATTAAA

      2830       2840       2850       2860       2870       2880
TTTTTATTTA TGCAGAGGCC GAGGCCGCCT CGGCCTCTGA GCTATTCCAG AAGTAGTGAG
AAAAATAAAT ACGTCTCCGG CTCCGGCGGA GCCGGAGACT CGATAAGGTC TTCATCACTC

      2890       2900       2910       2920       2930       2940
GAGGCTTTTT TGGAGGCCTA GGCTTTTGCA AAAAGCTTGG ACAGCTCAGG GCTGCGATTT
CTCCGAAAAA ACCTCCGGAT CCGAAAACGT TTTTCGAACC TGTCGAGTCC CGACGCTAAA

      2950       2960       2970       2980       2990       3000
CGCGCCAAAC TTGACGGCAA TCCTAGCGTG AAGGCTGGTA GGATTTTATC CCCGCTGCCA
GCGCGGTTTG AACTGCCGTT AGGATCGCAC TTCCGACCAT CCTAAAATAG GGGCGACGGT
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FIGURE 19F

pD17-hG1b

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      3010       3020       3030       3040       3050       3060
TCATGGTTCG ACCATTGAAC TGCATCGTCG CCGTGTCCCA AAATATGGGG ATTGGCAAGA
AGTACCAAGC TGGTAACTTG ACGTAGCAGC GGCACAGGGT TTTATACCCC TAACCGTTCT

      3070       3080       3090       3100       3110       3120
ACGGAGACCT ACCCTGGCCT CCGCTCAGGA ACGAGTTCAA GTACTTCCAA AGAATGACCA
TGCCTCTGGA TGGGACCGGA GGCGAGTCCT TGCTCAAGTT CATGAAGGTT TCTTACTGGT

      3130       3140       3150       3160       3170       3180
CAACCTCTTC AGTGGAAGGT AAACAGAATC TGGTGATTAT GGGTAGGAAA ACCTGGTTCT
GTTGGAGAAG TCACCTTCCA TTTGTCTTAG ACCACTAATA CCCATCCTTT TGGACCAAGA

      3190       3200       3210       3220       3230       3240
CCATTCCTGA GAAGAATCGA CCTTTAAAGG ACAGAATTAA TATAGTTCTC AGTAGAGAAC
GGTAAGGACT CTTCTTAGCT GGAAATTTCC TGTCTTAATT ATATCAAGAG TCATCTCTTG

      3250       3260       3270       3280       3290       3300
TCAAAGAACC ACCACGAGGA GCTCATTTTC TTGCCAAAAG TTTGGATGAT GCCTTAAGAC
AGTTTCTTGG TGGTGCTCCT CGAGTAAAAG AACGGTTTTC AAACCTACTA CGGAATTCTG

      3310       3320       3330       3340       3350       3360
TTATTGAACA ACCGGAATTG GCAAGTAAAG TAGACATGGT TTGGATAGTC GGAGGCAGTT
AATAACTTGT TGGCCTTAAC CGTTCATTTC ATCTGTACCA AACCTATCAG CCTCCGTCAA

      3370       3380       3390       3400       3410       3420
CTGTTTACCA GGAAGCCATG AATCAACCAG GCCACCTTAG ACTCTTTGTG ACAAGGATCA
GACAAATGGT CCTTCGGTAC TTAGTTGGTC CGGTGGAATC TGAGAAACAC TGTTCCTAGT

      3430       3440       3450       3460       3470       3480
TGCAGGAATT TGAAAGTGAC ACGTTTTTCC CAGAAATTGA TTTGGGGAAA TATAAACTTC
ACGTCCTTAA ACTTTCACTG TGCAAAAAGG GTCTTTAACT AAACCCCTTT ATATTTGAAG

      3490       3500       3510       3520       3530       3540
TCCCAGAATA CCCAGGCGTC CTCTCTGAGG TCCAGGAGGA AAAAGGCATC AAGTATAAGT
AGGGTCTTAT GGGTCCGCAG GAGAGACTCC AGGTCCTCCT TTTTCCGTAG TTCATATTCA

      3550       3560       3570       3580       3590       3600
TTGAAGTCTA CGAGAAGAAA GACTAACAGG AAGATGCTTT CAAGTTCTCT GCTCCCCTCC
AACTTCAGAT GCTCTTCTTT CTGATTGTCC TTCTACGAAA GTTCAAGAGA CGAGGGGAGG
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FIGURE 19G

pD17-hG1b

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      3610       3620       3630       3640       3650       3660
TAAAGCTATG CATTTTTATA AGACCATGGG ACTTTTGCTG GCTTTAGATC TCTTTGTGAA
ATTTCGATAC GTAAAAATAT TCTGGTACCC TGAAAACGAC CGAAATCTAG AGAAACACTT

      3670       3680       3690       3700       3710       3720
GGAACCTTAC TTCTGTGGTG TGACATAATT GGACAAACTA CCTACAGAGA TTTAAAGCTC
CCTTGGAATG AAGACACCAC ACTGTATTAA CCTGTTTGAT GGATGTCTCT AAATTTCGAG

      3730       3740       3750       3760       3770       3780
TAAGGTAAAT ATAAAATTTT TAAGTGTATA ATGTGTTAAA CTACTGATTC TAATTGTTTG
ATTCCATTTA TATTTTAAAA ATTCACATAT TACACAATTT GATGACTAAG ATTAACAAAC

      3790       3800       3810       3820       3830       3840
TGTATTTTAG ATTCCAACCT ATGGAACTGA TGAATGGGAG CAGTGGTGGA ATGCCTTTAA
ACATAAAATC TAAGGTTGGA TACCTTGACT ACTTACCCTC GTCACCACCT TACGGAAATT

      3850       3860       3870       3880       3890       3900
TGAGGAAAAC CTGTTTTGCT CAGAAGAAAT GCCATCTAGT GATGATGAGG CTACTGCTGA
ACTCCTTTTG GACAAAACGA GTCTTCTTTA CGGTAGATCA CTACTACTCC GATGACGACT

      3910       3920       3930       3940       3950       3960
CTCTCAACAT TCTACTCCTC CAAAAAAGAA GAGAAAGGTA GAAGACCCCA AGGACTTTCC
GAGAGTTGTA AGATGAGGAG GTTTTTTCTT CTCTTTCCAT CTTCTGGGGT TCCTGAAAGG

      3970       3980       3990       4000       4010       4020
TTCAGAATTG CTAAGTTTTT TGAGTCATGC TGTGTTTAGT AATAGAACTC TTGCTTGCTT
AAGTCTTAAC GATTCAAAAA ACTCAGTACG ACACAAATCA TTATCTTGAG AACGAACGAA

      4030       4040       4050       4060       4070       4080
TGCTATTTAC ACCACAAAGG AAAAAGCTGC ACTGCTATAC AAGAAAATTA TGGAAAAATA
ACGATAAATG TGGTGTTTCC TTTTTCGACG TGACGATATG TTCTTTTAAT ACCTTTTTAT

      4090       4100       4110       4120       4130       4140
TTCTGTAACC TTTATAAGTA GGCATAACAG TTATAATCAT AACATACTGT TTTTTCTTAC
AAGACATTGG AAATATTCAT CCGTATTGTC AATATTAGTA TTGTATGACA AAAAAGAATG

      4150       4160       4170       4180       4190       4200
TCCACACAGG CATAGAGTGT CTGCTATTAA TAACTATGCT CAAAAATTGT GTACCTTTAG
AGGTGTGTCC GTATCTCACA GACGATAATT ATTGATACGA GTTTTTAACA CATGGAAATC
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FIGURE 19H

pD17-hG1b

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      4210       4220       4230       4240       4250       4260
CTTTTTAATT TGTAAAGGGG TTAATAAGGA ATATTTGATG TATAGTGCCT TGACTAGAGA
GAAAAATTAA ACATTTCCCC AATTATTCCT TATAAACTAC ATATCACGGA ACTGATCTCT

      4270       4280       4290       4300       4310       4320
TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC TTTAAAAAAC CTCCCACACC
AGTATTAGTC GGTATGGTGT AAACATCTCC AAAATGAACG AAATTTTTTG GAGGGTGTGG

      4330       4340       4350       4360       4370       4380
TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG TTTATTGCAG
AGGGGGACTT GGACTTTGTA TTTTACTTAC GTTAACAACA ACAATTGAAC AAATAACGTC

      4390       4400       4410       4420       4430       4440
CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT
GAATATTACC AATGTTTATT TCGTTATCGT AGTGTTTAAA GTGTTTATTT CGTAAAAAAA

      4450       4460       4470       4480       4490       4500
CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGATCG
GTGACGTAAG ATCAACACCA AACAGGTTTG AGTAGTTACA TAGAATAGTA CAGACCTAGC

      4510       4520       4530       4540       4550       4560
GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCAACTTGT
CGACCTACTA GGAGGTCGCG CCCCTAGAGT ACGACCTCAA GAAGCGGGTG GGGTTGAACA

      4570       4580       4590       4600       4610       4620
TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG
AATAACGTCG AATATTACCA ATGTTTATTT CGTTATCGTA GTGTTTAAAG TGTTTATTTC

      4630       4640       4650       4660       4670       4680
CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG
GTAAAAAAAG TGACGTAAGA TCAACACCAA ACAGGTTTGA GTAGTTACAT AGAATAGTAC

      4690       4700       4710       4720       4730       4740
TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG
AGACATATGG CAGCTGGAGA TCGATCTCGA ACCGCATTAG TACCAGTATC GACAAAGGAC

      4750       4760       4770       4780       4790       4800
TGTGAAATTG TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA
ACACTTTAAC AATAGGCGAG TGTTAAGGTG TGTTGTATGC TCGGCCTTCG TATTTCACAT
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FIGURE 19I

pD17-hG1b

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      4810       4820       4830       4840       4850       4860
AAGCCTGGGG TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG
TTCGGACCCC ACGGATTACT CACTCGATTG AGTGTAATTA ACGCAACGCG AGTGACGGGC

      4870       4880       4890       4900       4910       4920
CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA
GAAAGGTCAG CCCTTTGGAC AGCACGGTCG ACGTAATTAC TTAGCCGGTT GCGCGCCCCT

      4930       4940       4950       4960       4970       4980
GAGGCGGTTT GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG
CTCCGCCAAA CGCATAACCC GCGAGAAGGC GAAGGAGCGA GTGACTGAGC GACGCGAGCC

      4990       5000       5010       5020       5030       5040
TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG
AGCAAGCCGA CGCCGCTCGC CATAGTCGAG TGAGTTTCCG CCATTATGCC AATAGGTGTC

      5050       5060       5070       5080       5090       5100
AATCAGGGGA TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC
TTAGTCCCCT ATTGCGTCCT TTCTTGTACA CTCGTTTTCC GGTCGTTTTC CGGTCCTTGG

      5110       5120       5130       5140       5150       5160
GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA
CATTTTTCCG GCGCAACGAC CGCAAAAAGG TATCCGAGGC GGGGGGACTG CTCGTAGTGT

      5170       5180       5190       5200       5210       5220
AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT
TTTTAGCTGC GAGTTCAGTC TCCACCGCTT TGGGCTGTCC TGATATTTCT ATGGTCCGCA

      5230       5240       5250       5260       5270       5280
TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC
AAGGGGGACC TTCGAGGGAG CACGCGAGAG GACAAGGCTG GGACGGCGAA TGGCCTATGG

      5290       5300       5310       5320       5330       5340
TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC
ACAGGCGGAA AGAGGGAAGC CCTTCGCACC GCGAAAGAGT TACGAGTGCG ACATCCATAG

      5350       5360       5370       5380       5390       5400
TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC
AGTCAAGCCA CATCCAGCAA GCGAGGTTCG ACCCGACACA CGTGCTTGGG GGGCAAGTCG
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FIGURE 19J

pD17-hG1b

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      5410       5420       5430       5440       5450       5460
CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT
GGCTGGCGAC GCGGAATAGG CCATTGATAG CAGAACTCAG GTTGGGCCAT TCTGTGCTGA

      5470       5480       5490       5500       5510       5520
TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG
ATAGCGGTGA CCGTCGTCGG TGACCATTGT CCTAATCGTC TCGCTCCATA CATCCGCCAC

      5530       5540       5550       5560       5570       5580
CTACAGAGTT CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA
GATGTCTCAA GAACTTCACC ACCGGATTGA TGCCGATGTG ATCTTCCTGT CATAAACCAT

      5590       5600       5610       5620       5630       5640
TCTGCGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA
AGACGCGAGA CGACTTCGGT CAATGGAAGC CTTTTTCTCA ACCATCGAGA ACTAGGCCGT

      5650       5660       5670       5680       5690       5700
AACAAACCAC CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA
TTGTTTGGTG GCGACCATCG CCACCAAAAA AACAAACGTT CGTCGTCTAA TGCGCGTCTT

      5710       5720       5730       5740       5750       5760
AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG
TTTTTCCTAG AGTTCTTCTA GGAAACTAGA AAAGATGCCC CAGACTGCGA GTCACCTTGC

      5770       5780       5790       5800       5810       5820
AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC
TTTTGAGTGC AATTCCCTAA AACCAGTACT CTAATAGTTT TTCCTAGAAG TGGATCTAGG

      5830       5840       5850       5860       5870       5880
TTTTAAATTA AAAATGAAGT TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG
AAAATTTAAT TTTTACTTCA AAATTTAGTT AGATTTCATA TATACTCATT TGAACCAGAC

      5890       5900       5910       5920       5930       5940
ACAGTTACCA ATGCTTAATC AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT
TGTCAATGGT TACGAATTAG TCACTCCGTG GATAGAGTCG CTAGACAGAT AAAGCAAGTA

      5950       5960       5970       5980       5990       6000
CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG
GGTATCAACG GACTGAGGGG CAGCACATCT ATTGATGCTA TGCCCTCCCG AATGGTAGAC
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FIGURE 19K

pD17-hG1b

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      6010       6020       6030       6040       6050       6060
GCCCCAGTGC TGCAATGATA CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA
CGGGGTCACG ACGTTACTAT GGCGCTCTGG GTGCGAGTGG CCGAGGTCTA AATAGTCGTT

      6070       6080       6090       6100       6110       6120
TAAACCAGCC AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA
ATTTGGTCGG TCGGCCTTCC CGGCTCGCGT CTTCACCAGG ACGTTGAAAT AGGCGGAGGT

      6130       6140       6150       6160       6170       6180
TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC
AGGTCAGATA ATTAACAACG GCCCTTCGAT CTCATTCATC AAGCGGTCAA TTATCAAACG

      6190       6200       6210       6220       6230       6240
GCAACGTTGT TGCCATTGCT ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT
CGTTGCAACA ACGGTAACGA TGTCCGTAGC ACCACAGTGC GAGCAGCAAA CCATACCGAA

      6250       6260       6270       6280       6290       6300
CATTCAGCTC CGGTTCCCAA CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA
GTAAGTCGAG GCCAAGGGTT GCTAGTTCCG CTCAATGTAC TAGGGGGTAC AACACGTTTT

      6310       6320       6330       6340       6350       6360
AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT
TTCGCCAATC GAGGAAGCCA GGAGGCTAGC AACAGTCTTC ATTCAACCGG CGTCACAATA

      6370       6380       6390       6400       6410       6420
CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT
GTGAGTACCA ATACCGTCGT GACGTATTAA GAGAATGACA GTACGGTAGG CATTCTACGA

      6430       6440       6450       6460       6470       6480
TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA
AAAGACACTG ACCACTCATG AGTTGGTTCA GTAAGACTCT TATCACATAC GCCGCTGGCT

      6490       6500       6510       6520       6530       6540
GTTGCTCTTG CCCGGCGTCA ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG
CAACGAGAAC GGGCCGCAGT TATGCCCTAT TATGGCGCGG TGTATCGTCT TGAAATTTTC

      6550       6560       6570       6580       6590       6600
TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA
ACGAGTAGTA ACCTTTTGCA AGAAGCCCCG CTTTTGAGAG TTCCTAGAAT GGCGACAACT
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FIGURE 19L

pD17-hG1b

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      6610       6620       6630       6640       6650       6660
GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA
CTAGGTCAAG CTACATTGGG TGAGCACGTG GGTTGACTAG AAGTCGTAGA AAATGAAAGT

      6670       6680       6690       6700       6710       6720
CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG
GGTCGCAAAG ACCCACTCGT TTTTGTCCTT CCGTTTTACG GCGTTTTTTC CCTTATTCCC

      6730       6740       6750       6760       6770       6780
CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC
GCTGTGCCTT TACAACTTAT GAGTATGAGA AGGAAAAAGT TATAATAACT TCGTAAATAG

      6790       6800       6810       6820       6830       6840
AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG
TCCCAATAAC AGAGTACTCG CCTATGTATA AACTTACATA AATCTTTTTA TTTGTTTATC

      6850       6860       6870       6880       6890       6900
GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT CGACGGATCG GGAGATCTGC
CCCAAGGCGC GTGTAAAGGG GCTTTTCACG GTGGACTGCA GCTGCCTAGC CCTCTAGACG

      6910       6920       6930       6940       6950       6960
TAGGTGACCT GAGGCGCGCC GGCTTCGAAT AGCCAGAGTA ACCTTTTTTT TTAATTTTAT
ATCCACTGGA CTCCGCGCGG CCGAAGCTTA TCGGTCTCAT TGGAAAAAAA AATTAAAATA

      6970       6980       6990       7000       7010       7020
TTTATTTTAT TTTTGAGATG GAGTTTGGCG CCGATCTCCC GATCCCCTAT GGTCGACTCT
AAATAAAATA AAAACTCTAC CTCAAACCGC GGCTAGAGGG CTAGGGGATA CCAGCTGAGA

      7030       7040       7050       7060       7070       7080
CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT
GTCATGTTAG ACGAGACTAC GGCGTATCAA TTCGGTCATA GACGAGGGAC GAACACACAA

      7090       7100       7110       7120       7130       7140
GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA
CCTCCAGCGA CTCATCACGC GCTCGTTTTA AATTCGATGT TGTTCCGTTC CGAACTGGCT

      7150       7160       7170       7180       7190       7200
CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC
GTTAACGTAC TTCTTAGACG AATCCCAATC CGCAAAACGC GACGAAGCGC TACATGCCCG
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FIGURE 19M

pD17-hG1b

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      7210       7220       7230       7240       7250       7260
CAGATATACG CGTTGACATT GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC
GTCTATATGC GCAACTGTAA CTAATAACTG ATCAATAATT ATCATTAGTT AATGCCCCAG

      7270       7280       7290       7300       7310       7320
ATTAGTTCAT AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC
TAATCAAGTA TCGGGTATAT ACCTCAAGGC GCAATGTATT GAATGCCATT TACCGGGCGG

      7330       7340       7350       7360       7370       7380
TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT
ACCGACTGGC GGGTTGCTGG GGGCGGGTAA CTGCAGTTAT TACTGCATAC AAGGGTATCA

      7390       7400       7410       7420       7430       7440
AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA
TTGCGGTTAT CCCTGAAAGG TAACTGCAGT TACCCACCTG ATAAATGCCA TTTGACGGGT

      7450       7460       7470       7480       7490       7500
CTTGGCAGTA CATCAAGTGT ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG
GAACCGTCAT GTAGTTCACA TAGTATACGG TTCATGCGGG GGATAACTGC AGTTACTGCC

      7510       7520       7530       7540       7550       7560
TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA
ATTTACCGGG CGGACCGTAA TACGGGTCAT GTACTGGAAT ACCCTGAAAG GATGAACCGT

      7570       7580       7590       7600       7610       7620
GTACATCTAC GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA
CATGTAGATG CATAATCAGT AGCGATAATG GTACCACTAC GCCAAAACCG TCATGTAGTT

      7630       7640       7650       7660       7670       7680
TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA
ACCCGCACCT ATCGCCAAAC TGAGTGCCCC TAAAGGTTCA GAGGTGGGGT AACTGCAGTT

      7690       7700       7710       7720       7730       7740
TGGGAGTTTG TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC
ACCCTCAAAC AAAACCGTGG TTTTAGTTGC CCTGAAAGGT TTTACAGCAT TGTTGAGGCG

      7750       7760       7770       7780       7790       7800
CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT
GGGTAACTGC GTTTACCCGC CATCCGCACA TGCCACCCTC CAGATATATT CGTCTCGAGA
```

FIGURE 19N

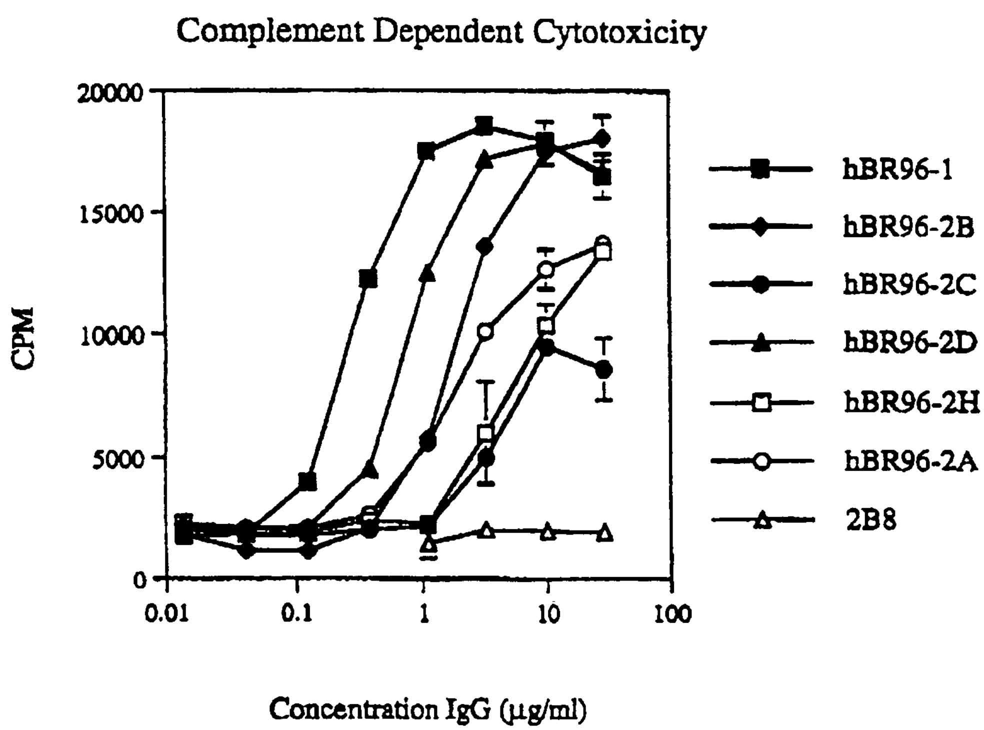
pD17-hG1b

```
      7810       7820       7830       7840       7850       7860
CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
GACCGATTGA TCTCTTGGGT GACGAATGAC CGAATAGCTT TAATTATGCT GAGTGATATC

      7870       7880
GGAGACCCAA GCTT
CCTCTGGGTT CGAA
```

47156

FIGURE 20



48156

FIGURE 21

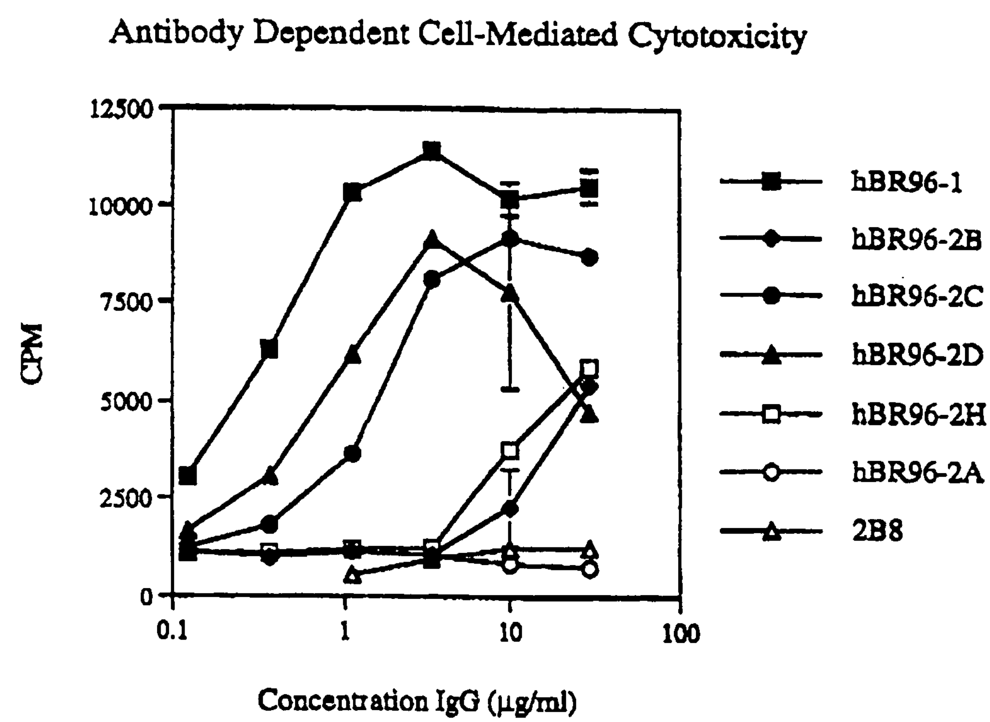
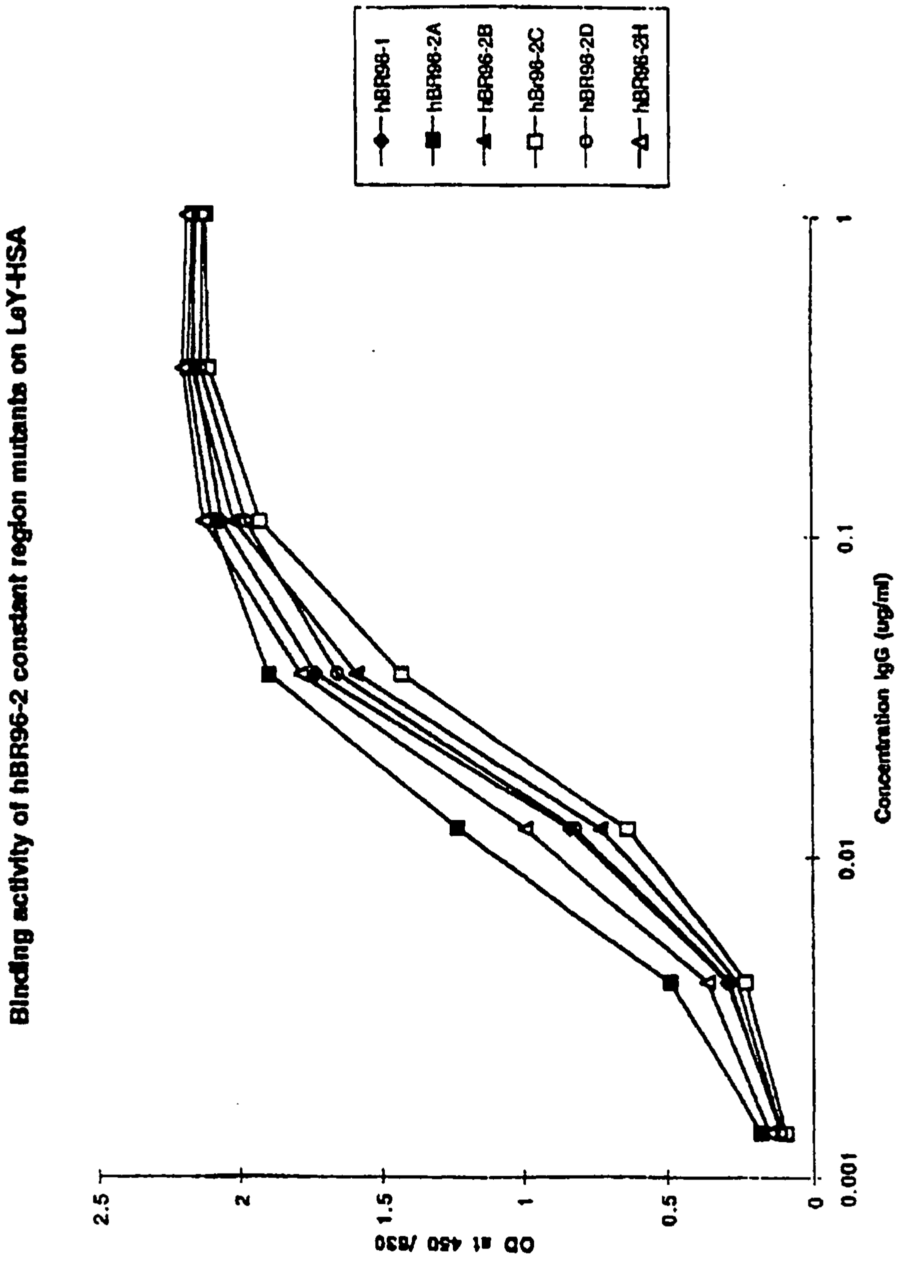
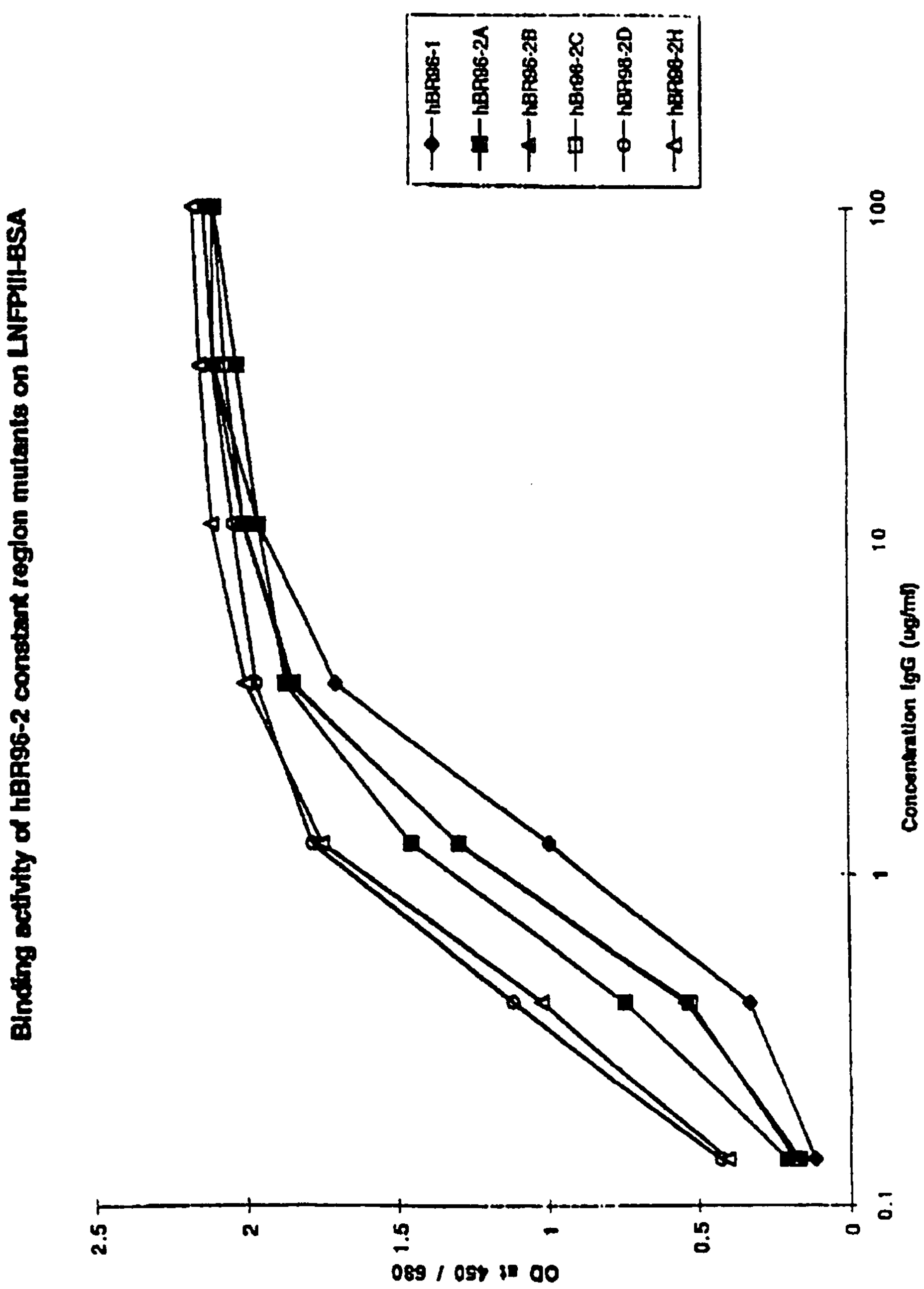

Antibody Dependent Cell-Mediated Cytotoxicity
12500
10000
7500
5000
2500
0
CPM
0.1
1
10
100
Concentration IgG (µg/ml)
hBR96-1
hBR96-2B
hBR96-2C
hBR96-2D
hBR96-2H
hBR96-2A
2B8

FIGURE 22



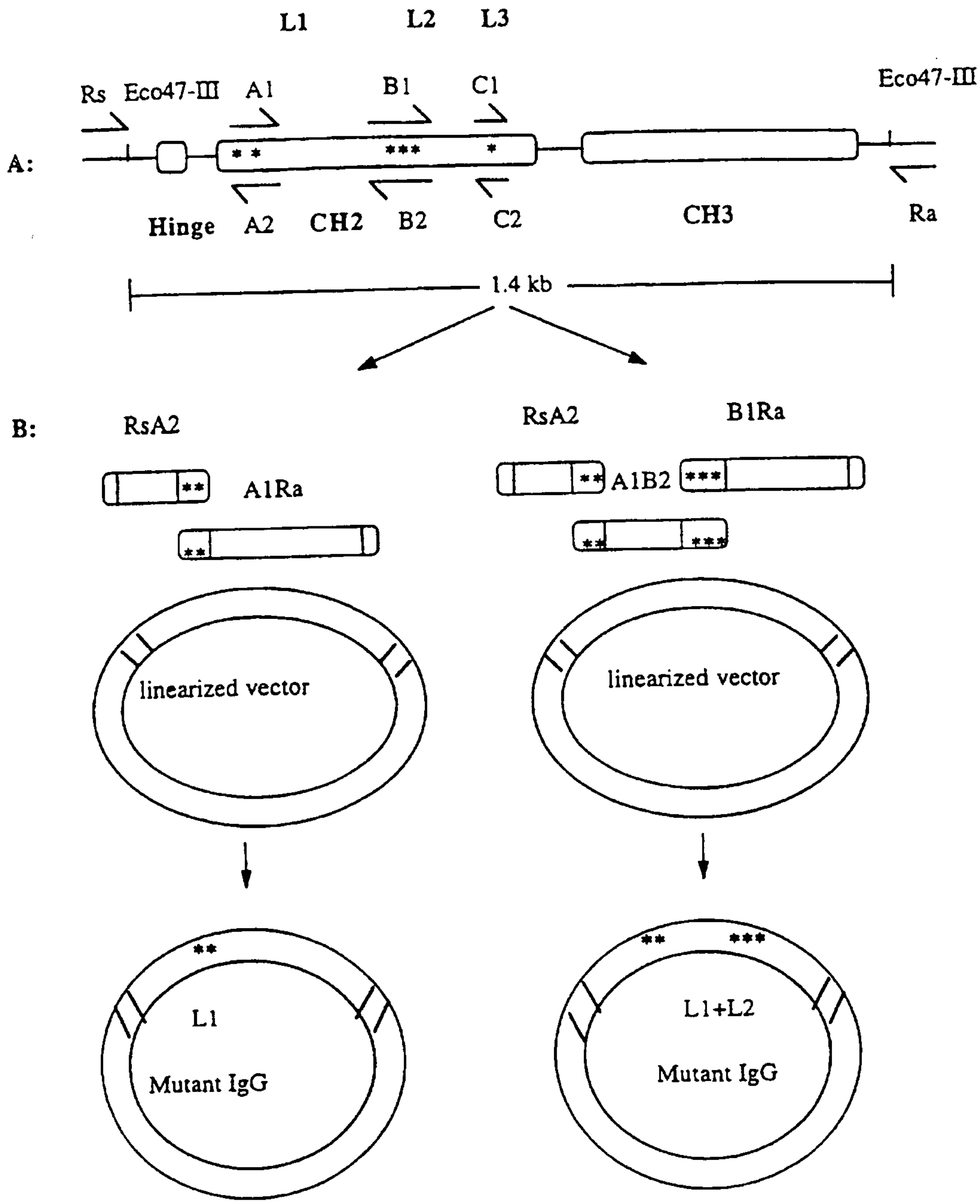
50156

FIGURE 23



51156

Figure 24



52156

Figure 25

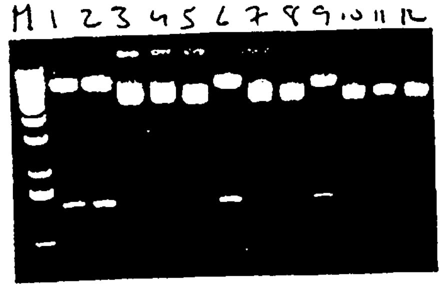


Figure 26

hBR96-2 Heavy Chain Variable Region (V_H)

1 EVQLVESGGG 11 LVQPGGSLRL 21 SCAASGFPFS 31 DYYMYWVRQA 41 PGKGLEWVSY

51 ISQDGDITDY 61 ADSVKGRFTI 71 SRDNAKNSLY 81 LQMNSLRDED 91 TAVYYCARGL

101 ADGAWFAYWG 111 QGTLVTVSS

human IgG1 constant

CH1
A STKGPSVFPL APSSKSTSGG TAALGCLVKD

YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY

CH2 235 237
ICNVNHKPSN TKVDKKVEPK SCDKTHTCPP CPAPELLGGP SVFLFPPKPK

DTLMISRTPE VTCVVVDVSH EDPEVKFNWY VDGVEVHNAK TKPREEQYNS

318 320 322 331 CH3
TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKTISK AKGQPREPQV

YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTPPVL

DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSPGK

Figure 27

hBR96-2A: Heavy Chain Variable Region (V_H)

1 EVQLVESGGG 11 LVQPGGSLRL 21 SCAASGFPFS 31 DYYMYWVRQA 41 PGKGLEWVSY

51 ISQDGDITDY 61 ADSVKGRFTI 71 SRDNAKNSLY 81 LQMNSLRDED 91 TAVYYCARGL

101 ADGAWFAYWG 111 QGTLVTVSS

hBR96-2A: Human Heavy Chain IgG1 Constant Region ΔCH2

A STKGPSVFPL APSSKSTSGG TAALGCLVKD YFPEPVTVSW NSGALTSGVH

TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY ICNVNHKPSN TKVDKKVEPK

SCDKTHTCPP CP GQPREPQV YTLPPSRDEL TKNQVSLTCL VKGFYPSDIA

VEWESNGQPE NNYKTTPPVL DSDGSFFLYS KLTVDKSRWQ QGNVFSCSVM

HEALHNHYTQ KSLSLSPGK

Figure 28

This sequence is the chi BR96 IgG1 with CH2 deleted.

VH

1 EVNLVESGGG LVQPGGSLKV SCVTSGFTFS DYYMYWVRQT PEKRLEWVAY

51 ISQGGDITDY PDTVKGRFTI SRDNAKNTLY LQMSRLKSED TAMYYCARGL

CH1

101 DDGAWFAYWG QGTLVTVSVA STKGPSVFPL APSSKSTSGG TAALGCLVKD

151 YFPEPVTVSW NSGALTSGVH TFPAVLQSSG LYSLSSVVTV PSSSLGTQTY

CH3

201 ICNVNHKPSN TKVDKKVEPK SCDKTHTCPP CHGQPREPQV YTLPPSRDEL

251 TKNQVSLTCL VKGFYPSDIA VEWESNGQPE NNYKTTPPVL DSDGSFFLYS

301 KLTVDKSRWQ QGNVFSCSVM HEALHNHYTQ KSLSLSPGK

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/13562

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 A61K39/395 A61K38/17 A61K47/48 A61K51/10
C07K16/30 C07K16/46 C07K16/00 C12N15/13 C12N1/21
C12N5/10 //C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. GILLIES ET AL.: "Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumor specificities." HUMAN ANTIBODIES AND HYBRIDOMAS, vol. 1, no. 1, 1990, STONEHAM, MA, USA, pages 47-54, XP002050448 see the whole document ---	1-8, 23-25

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1997	21.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. SCHREIBER ET AL.: "An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice." CANCER RESEARCH, vol. 52, no. 12, 15 June 1992, BALTIMORE, MD, USA, pages 3262-3266, XP002050449 see abstract	33,35,36
A		1,2,5,7, 8,11-18, 23
A	A. DUNCAN ET AL.: "The binding site for Clq on IgG." NATURE, vol. 332, no. 6166, 21 April 1988, LONDON, GB, pages 738-740, XP002050450 cited in the application see the whole document	1,2,5,7, 8
A	J. LUND ET AL.: "Human FcgammaRI and FcgammaRII interact with distinct but overlapping sites on human IgG." THE JOURNAL OF IMMUNOLOGY, vol. 147, no. 8, 15 October 1991, BALTIMORE, MD, USA, pages 2657-2662, XP002050451 cited in the application see abstract	1,2,5,7, 8
A	Y. XU ET AL.: "Residue at position 331 in the IgG1 and IgG4 CH2 domains contributes to their differential ability to bind and activate complement." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 5, 4 February 1994, BALTIMORE, MD, USA, pages 3469-3474, XP002050452 cited in the application see abstract see discussion	1-8
A	T. MICHAELSEN ET AL.: "One disulfide bond in front of the second heavy chain constant region is necessary and sufficient for effector functions of human IgG3 without a genetic hinge." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 91, no. 20, 27 September 1994, WASHINGTON, DC, USA, pages 9243-9247, XP002050453 see the whole document	1,2,5,7, 8

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L. TAN ET AL.: "Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 87, no. 1, January 1990, WASHINGTON, DC, USA, pages 162-166, XP002050454 see the whole document ---	1-8
A	EP 0 699 756 A (BRISTOL-MYERS SQUIBB COMPANY) 6 March 1996 cited in the application see examples see claims -----	11-18, 23,25, 28,29, 31-52

1

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US 97/13562

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/13562

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 26,27

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 26 represents a method of detection/diagnosis and refers forward to claim 30, which represents a method of treatment. Claim 27 refers to a method in claim 24; however, in claim 24 a product is claimed, not a method.

Remark : Although claims 1-22, 25, 28-32 and 34-36 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/13562

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 699756 A	06-03-96	AU 2834995 A	15-02-96
		CA 2155397 A	05-02-96
		JP 8191692 A	30-07-96